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# Mutational Analysis of Geopilin Function in *Geobacter Sulfurreducens*

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**MUTATIONAL ANALYSIS OF GEOPILIN FUNCTION IN *GEOBACTER*  
*SULFURREDUCTENS***

A Dissertation Presented

by

LUBNA V. RICHTER

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfillment  
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MAY 2011

Department of Chemistry

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**MUTATIONAL ANALYSIS OF GEOPILIN FUNCTION IN *GEOBACTER*  
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## **DEDICATION**

To the one I witnessed the miracle of life with

The one who inspired me by the beauty of simplicity

The one who stood by my side when I needed a shoulder to lean on

The one who proved to me that love speaks all languages and believes in no boundaries

To my best friend, my soulmate, my Hanno

I dedicate this work

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## **ABSTRACT**

### MUTATIONAL ANALYSIS OF GEOPILIN FUNCTION IN *GEOBACTER* *SULFURREDUCTENS*

MAY 2011

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*Geobacter sulfurreducens* possesses type IV pili that are considered to be conductive nanowires and a crucial structural element in biofilm formation, enabling electron transfer to insoluble metal oxides in anaerobic sediments and to graphite anodes in microbial fuel cells. The molecular mechanism by which electrons are transferred through the nanowires to the electron acceptor is not fully understood. Prior to the work described in this thesis, the gene (*pilA*) encoding the structural pilus subunit had been identified, but little was known about the functional translation start codon, the length of the mature secreted protein, or what renders the pili conductive.

Using mass spectrometry, I found that a tyrosine residue (Y32) near the carboxyl terminus of the mature PilA protein is posttranslationally modified by attachment of glycerophosphate. I studied the significance of Y32 for biofilm formation on various surfaces and for growth of *G. sulfurreducens* with insoluble electron acceptors. A mutant in which Y32 was replaced by phenylalanine lacked the glycerophosphate; biofilm formation on graphite surfaces was severely diminished and current production in microbial fuel cells was initiated only after a long lag phase. Moreover, cells with Y32F mutation in the *pilA* gene exhibited growth deficiency when Fe(III) oxide was the sole



electron acceptor. My data confirm the role of *G. sulfurreducens* pili in biofilm formation and electron transfer to Fe(III) oxide and identify an amino acid in the PilA protein that is essential for these two processes.

I also confirmed the existence of two functional translation start codons for the *pilA* gene and identified two isoforms (short and long) of the PilA preprotein by series of genetic complementation experiments. The short PilA isoform is found predominantly in an intracellular fraction, and seems to stabilize the long isoform and influence the secretion of several outer surface *c*-type cytochromes. The long PilA isoform, on the other hand, is required for secretion of PilA to the outer surface of the cell, a process that requires co-expression of *pilA* and the nine genes on its 3' side. The long isoform is essential for biofilm formation on various surfaces, for optimum current production in microbial fuel cells, and for growth on insoluble Fe(III) oxide.

This study provides new insight concerning the function and biogenesis of *Geobacter* type IV PilA, as well as a foundation for further research that will be conducted on microbial nanowires.

## TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS .....	v
ABSTRACT .....	vii
LIST OF TABLES .....	xi
LIST OF FIGURES .....	xii
 CHAPTER	
1. INTRODUCTION .....	1
Type IV pilin: function, structure and assembly mechanism .....	1
<i>Geobacter</i> species; bioremediation and generation of electricity .....	5
Type IV pili of <i>Geobacter sulfurreducens</i> ; involvement in current production and Fe(III) reduction.....	10
2. THE ROLE OF TYROSINE-32 OF GEOPILIN IN BIOFILMS FORMATION AND GROWTH ON FE(III) OXIDE.....	14
Introduction.....	14
Material and methods.....	17
Results.....	30
Discussion.....	46
Summary and future directions .....	49
3. DIFFERENTIATION OF THE ROLES OF TWO ISOFORMS OF GEOPILIN PREPROTEIN IN <i>GEOBACTER SULFURREDUCENS</i> .....	50
Introduction.....	50
Material and methods.....	54
Results.....	61
Discussion.....	78
Summary and conclusions .....	83
4. INVESTIGATION OF THE GENES REQUIRED IN <i>CIS</i> FOR EXPRESSION AND SECRETION OF GEOPILIN .....	85
Intoduction .....	85
Materials and methods .....	88
Results.....	92
Summary and conclusions .....	107

5.	FUTURE STUDIES.....	109
	Amino acids important for conductivity and Fe(III) oxide reduction in the C-terminal portion of PilA .....	109
	Investigation of the role of GSU1497 in electron transfer to insoluble electron acceptors.....	112
APPENDICES		
A.	THE PLC1 PLASMID MAP, AND GROWTH CURVES OF DL1 HARBORING EITHER PRG5 OR PLC1.....	116
B.	LOCALIZATION OF THE PILA PROTEIN IN CELL CULTURE .....	118
C.	MATRIX-ASSISTED LASER DESORPTION/IONIZATION (MALDI) MASS SPECTROMETRIC DATA OBSERVED FOR THE TRYPTIC DIGESTED PEPTIDE (AYNSAASSDLR) OF THE PILA PROTEIN SECRETED BY <i>G. SULFURREDUCTENS</i> .....	119
D.	PROTEIN SEQUENCE ALIGNMENT OF GSU1496, TYPE IV PILIN OF <i>G. SULFURREDUCTENS</i> , AND OTHER TYPE IV PILINS FROM GRAM-NEGATIVE BACTERIA .....	121
E.	LIST OF PLASMIDS GENERATED DURING THE PH.D. RESEARCH COURSE BUT NOT USED IN ANY OF THE CHAPTERS .....	130
	BIBLIOGRAPHY .....	131

## LIST OF TABLES

Table	Page
1. <i>Geobacter sulfurreducens</i> strains and plasmids used in Chapter 2 .....	17
2. List of primers used in Chapter 2.....	20
3. Peptides detected by MALDI MS/MS of the tryptic-digested mature and secreted PilA .....	30
4. <i>Geobacter sulfurreducens</i> strains and plasmids used in Chapter 3 .....	54
5. List of primers used in Chapter 3.....	56
6. Quantitative analysis of the mature biofilms of the wildtype DL100 and the short isoform pilA4(M1) strains grown on poised graphite electrodes in microbial fuel cells and producing maximum current for three days .....	73
7. <i>Geobacter sulfurreducens</i> strains and plasmids used in Chapter 4 .....	88
8. List of primers used in Chapter 4.....	90
9. Comparison of the transcriptional levels of pilA, GSU1497 and the 3' end gene cluster under various growth conditions .....	101

## LIST OF FIGURES

Figure	Page
1. A model of type IV pilus assembly.....	4
2. Light microscopic image of <i>Geobacter sulfurreducens</i> cells colonizing a Fe(III) oxide particle.....	6
3. Ability of <i>Geobacter</i> species to couple oxidation of organic compounds to reduction of metals.....	6
4. Scanning electron microscopic (SEM) images of the mature thick biofilm of <i>Geobacter sulfurreducens</i> wild type (DL1) strain grown on a poised graphite electrode.....	8
5. Genomic organization of the pilus biogenesis genes and the 3' side genes surrounding the <i>pilA</i> gene of <i>Geobacter sulfurreducens</i> .....	12
6. Method of PilA protein sample fractionation and preparation .....	27
7. Amino acid sequence of the PilA protein (GSU1496).....	32
8. Characterization of the <i>pilA3</i> (Y32F) mutant strain .....	35
9. Confocal laser scanning microscopic (CLSM) images of the <i>pilA3</i> (Y32F) biofilm on glass and graphite surfaces.....	37
10. Average biomass of the <i>pilA3</i> (Y32F) mutant strain on glass and graphite surfaces .....	38
11. Current production by the wildtype DL100 (PilA <sup>+</sup> ) and the <i>pilA3</i> (Y32F) mutant (PilAY32F) strains in microbial fuel cells.....	40
12. Confocal scanning micrographs of the wildtype DL100 (PilA <sup>+</sup> ) and the <i>pilA3</i> (Y32F) mutant (PilAY32F) biofilms formed on graphite anodes and producing maximum current for 3 days in microbial fuel cells.....	41
13. Reduction of insoluble Fe(III) oxide by the wildtype DL100 (PilA <sup>+</sup> ) and the <i>pilA3</i> (Y32F) mutant (PilAY32F) strains.....	42
14. Confocal microscopic analysis of the wildtype DL100 (A and D), <i>pilA3</i> (Y32F) (B and E) and $\Delta$ ( <i>pilA</i> )2 (C and F) biofilms formed on Fe(III) oxide-coated glass .....	44

15. Average cell growth of the wildtype DL100 (PilA <sup>+</sup> ), the <i>pilA3</i> (Y32F) (PilAY32F) and the $\Delta$ ( <i>pilA</i> )2 (PilA <sup>-</sup> ) biofilms formed on Fe(III) oxide-coated glass surfaces .....	45
16. Analysis of the nucleotide sequence of the <i>pilA</i> gene and the 5' region .....	53
17. Illustration of the <i>G. sulfurreducens</i> mutant strains used in Chapter 3 .....	63
18. Qualitative reverse transcriptase PCR reactions of the wildtype and the mutant strains .....	64
19. Characterization of the <i>pilA4</i> (M1), <i>pilA5</i> (RBS) and <i>pilA7</i> mutant strains .....	67
20. Western blot analysis of the <i>pilA6</i> (G20L) mutant strain .....	69
21. Current production by the wildtype DL100 (PilA <sup>+</sup> ) and the <i>pilA4</i> (M1) mutant (PilAM1) strains in microbial fuel cells .....	72
22. Confocal scanning micrographs of the isogenic wildtype DL100 (PilA <sup>+</sup> ) and the <i>pilA4</i> (M1) (PilAM1) biofilms formed on graphite anodes and producing maximum current for 3 days in microbial fuel cells.....	73
23. Average biomass of the mature wildtype DL100 (PilA <sup>+</sup> ), the <i>pilA4</i> (M1) (PilAM1) and the $\Delta$ ( <i>pilA</i> )2 (PilA <sup>-</sup> ) biofilms formed on glass (solid bars) and graphite (open bars) surfaces .....	75
24. Confocal microscopic analyses of the wildtype DL100 (A and D), the <i>pilA4</i> (M1) (B and E) and the $\Delta$ ( <i>pilA</i> )2 (C and F) biofilms formed on Fe(III) oxide-coated glass .....	77
25. Average cell growth of the wildtype (PilA <sup>+</sup> ), the <i>pilA4</i> (M1) (PilAM1) and the $\Delta$ ( <i>pilA</i> )2 (PilA <sup>-</sup> ) biofilms formed on Fe(III) oxide-coated glass surfaces .....	78
26. Models proposed for assembly and function of the short and long PilA preprotein isoforms .....	84
27. Illustration of the strategy used for construction of the <i>pilA</i> expression plasmid pLC19.....	91
28. Illustration of gene organization in the mutant strains used in Chapter 4 .....	94
29. Expression of the <i>pilA</i> gene from different plasmids in the $\Delta$ ( <i>pilA</i> )2 mutant strain.....	95

30. Comparison of outer membrane <i>c</i> -type cytochrome distribution when the entire <i>pilA</i> vs. an N-terminally truncated sequence of <i>pilA</i> is expressed .....	96
31. Characterization of the $\Delta$ GSU1497 strain .....	98
32. Illustration of the DNA sequence cloned in each of the plasmids used for complementation in this work .....	100
33. Expression of the <i>pilA</i> gene from different plasmids in the <i>pilA4</i> (M1) mutant strain .....	104
34. Expression of the <i>pilA</i> gene from different plasmids in the <i>pilA5</i> (RBS) mutant strain .....	105
35. Attachment assay of the <i>pilA4</i> (M1)_pLC19 strain to glass surfaces .....	106
36. DNA gel of the qualitative reverse transcriptase PCR reactions of $\Delta$ ( <i>pilA</i> )2_pLC3 .....	114
37. Predicted secondary structure of GSU1496 (PilA) and GSU1497 using I-TASSER.....	115
38. Amino acid sequence of the GSU1497 protein.....	115

# CHAPTER 1

## INTRODUCTION

### **Type IV pilin: function, structure and assembly mechanism**

Type IV pili are hairlike surface appendages expressed by both Gram-negative and Gram-positive bacteria of medical and environmental importance (Mattick, 2002; Telford et al., 2006). They are extremely thin (5-8 nm in diameter) but can extend up to several micrometers in length. They have different functions such as attachment to surfaces, DNA uptake during transformation, biofilm formation and twitching motility (Shi et al., 1996; Watson et al., 1996; O'Toole and Kolter, 1998; Ward and Zusman, 1999; Aas et al., 2002; Averhoff and Friedrich, 2003).

Type IV pili are composed primarily of multiple copies of the same protein subunit, usually termed pilin or PilA, which are arrayed as a filamentous helical polymer and which may be glycosylated and/or phosphorylated in different bacterial species (Soto and Hultgren, 1999; Mattick, 2002; Aas et al., 2006).

The pilin proteins are synthesized as precursors (prepilins) with a hydrophobic signal recognition peptide ending with a glycine residue (Strom and Lory, 1991). The signal peptide is responsible for translocating the protein across the inner membrane (Arts et al., 2007; Francetic et al., 2007), where it gets cleaved after the conserved glycine (position -1) by a unique leader peptidase, an inner membrane-associated enzyme termed PilD (Strom and Lory, 1993; Lory and Strom, 1997). The type IV pilus biogenesis proteins can differ from one species to another except for some conserved proteins called the core proteins. The core proteins include several proteins with an N-terminal pilin-like motif (PilE, PilV, PilW and FimU), a specific peptidase (PilD) that processes the prepilin



and prepilin-like proteins, a traffic ATPase (PilB) that powers the process of pilus assembly, a polytopic inner membrane protein (PilC), and a multimeric outer membrane protein named secretin (PilQ) that is necessary for the emergence of pili on the bacterial surface (Nunn et al., 1990; Hobbs and Mattick, 1993; Lauer et al., 1993; Martin et al., 1993; Strom and Lory, 1993; Alm and Mattick, 1997; Lory and Strom, 1997; Russel, 1998).

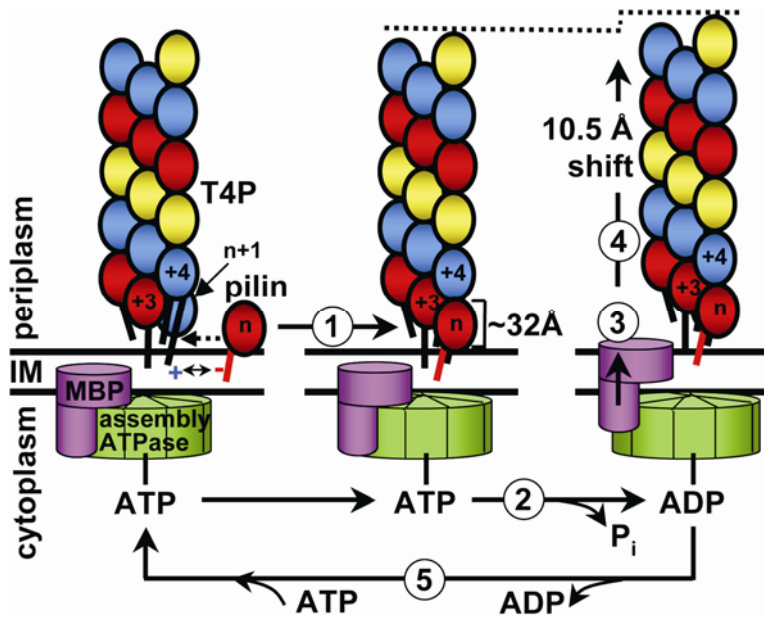
Type IV pilins are divided into two subclasses according to the lengths of the leader peptide and the mature protein. Type IVa pilins (e.g. pilins of *Pseudomonas aeruginosa* and *Neisseria meningitidis*) have short leader peptides (less than 10 aa), whereas type IVb pilins (e.g. pilins of Enteropathogenic *Escherichia coli* (EPEC) and *Vibrio cholerae*) exhibit long leader peptides (up to 30 aa) (Alm and Mattick, 1997; Ramer et al., 2002; Kirn et al., 2003; Carbonnelle et al., 2005; Carbonnelle et al., 2006). The two subgroups share similar features including the assembly pathway, sequence similarity in the N-terminus, but not in the C-terminus, and the type IV pilus biogenesis core proteins (Pugsley, 1993; Strom and Lory, 1993; Giron et al., 1997). However, the type IVa pilus biogenesis genes are scattered throughout the genome, whereas those of the type IVb subclass are always clustered (Pelicic, 2008).

The mature pilin subunits always display a highly hydrophobic amino-terminal segment with a consensus sequence that forms the core of the pilus fiber. The fifth residue is almost invariably a glutamate (E5), and substitution of this amino acid precludes type IV pilin expression and its associated functions (Pasloske et al., 1989; Strom and Lory, 1991; Macdonald et al., 1993; Horiuchi and Komano, 1998; Aas et al., 2002). The central and carboxyl-terminal segments of the pilin protein are relatively

hydrophilic and often stabilized by one or two disulfide bridges. The C-terminus folds into a four-stranded antiparallel  $\beta$ -sheet forming the “globular head” domain with negatively and positively charged patches exposed to the outside environment (Parge et al., 1995; Keizer et al., 2001; Craig et al., 2003; Audette et al., 2004; Craig et al., 2004; Xu et al., 2004; Craig et al., 2006). The charged residues exposed on the pilus surface are directly involved in the functions of the pilus in antigenicity, surface adhesion and microcolony formation (Mattick, 1993; Pugsley, 1993; Tennent, 1994; Parge et al., 1995; Hazes et al., 2000; Kirn et al., 2000; Keizer et al., 2001; Craig et al., 2003; Ramboarina et al., 2005). The sequence of the C-terminus varies according to the species and the pilus function except for the highly conserved serine-63 which is often modified posttranslationally by glycosylation (Stimson et al., 1996; Marceau et al., 1998; Power et al., 2000; Castric et al., 2001; Aas et al., 2006). The exposed Ser63-linked carbohydrates affect the interactions of the pilus with nearby pili, host receptors and antibodies (Power et al., 2003). Mutation of Ser63 to alanine eliminates glycosylation and increases pilus-bundling, suggesting that the glycoside modification is exposed and contributes to the chemistry of the pilus surface (Stimson et al., 1995; Marceau et al., 1998).

The accepted model for filament assembly (Craig et al., 2006) suggests that pilin subunits are added to the growing filament by electrostatic attraction between the side-chain of glutamate-5 of the incoming subunit and the methylamino group of *N*-methylphenylalanine-1 of the preceding subunit. The growing filament is comprised nine helices organized in three strands of three-helix bundles of pilin subunits. The growth of the filament is powered by the three active sites of the hexameric assembly ATPase positioned on the cytoplasmic side of the inner membrane. An ATP molecule bound by

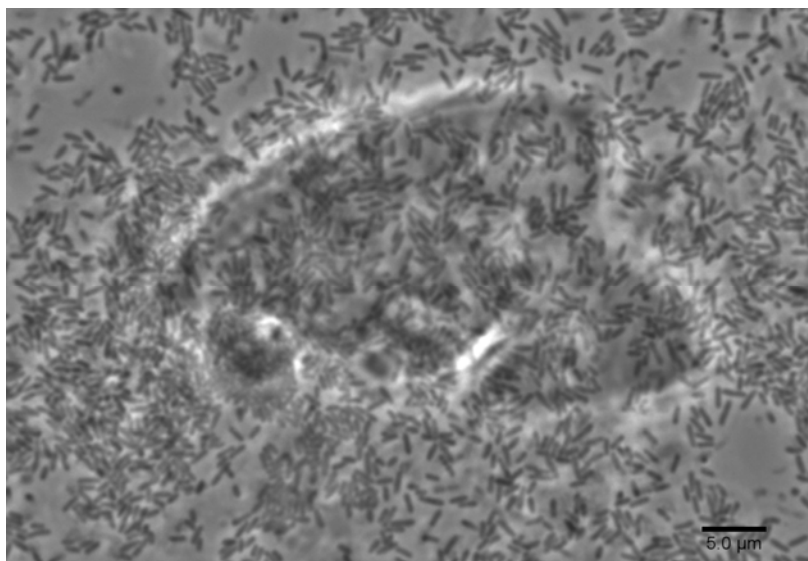
the ATPase is hydrolyzed, which induces a piston-like motion in the ATPase membrane-binding partner (MBP). The motion drives one of the three-helix bundle strands of the growing filament upward allowing the addition of a new pilin subunit. The ADP molecule is exchanged for ATP, allowing the assembly apparatus to relax to its resting position (Figure 1). The same mechanism is repeated for the other two ATPs bound by the hexameric ATPase complex, allowing the addition of two more pilin subunits to the remaining two strands in the three-helix bundle.



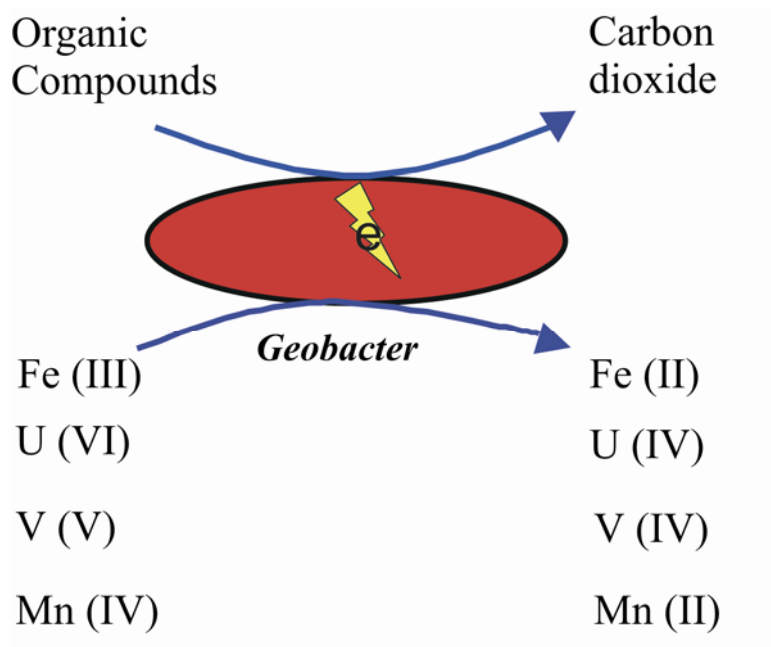
**Figure 1.** A model of the type IV pilus assembly. The growing three strands are colored in red, blue and yellow. The membrane-binding protein (MBP) is depicted in purple. Upon the electrostatic attraction between Glu5 of the incoming subunit and Phe1 of the existing subunit, one ATP molecule is hydrolyzed, which induces a piston-like motion in the MBP. This motion drives the growing strand upwards, allowing the addition of a new subunit. The figure source is Craig *et al.* 2006.

### **Geobacter species; bioremediation and generation of electricity**

*Geobacter* species are subsurface microorganisms of the *Deltaproteobacteria* class. They are of great interest due to their potential applications in environmental bioremediation and generation of electricity. *Geobacter* species can transfer electrons derived from oxidation of organic compounds to insoluble iron(III) oxide (Figure 2), which is ubiquitous in many subsurface environments, and also to a variety of other soluble and insoluble electron acceptors, including other metal oxide and fumarate (Figure 3) (Lovley and Phillips, 1988; Lovley, 1991b; Lovley et al., 1993; Caccavo et al., 1994; Childers et al., 2002; Butler et al., 2004; Lovley et al., 2004; Ortiz-Bernad et al., 2004; Afkar et al., 2005; Mehta et al., 2005; Holmes et al., 2007). *Geobacter* species are predominant and highly abundant in uranium-contaminated groundwater (over 90% of the microbial population) when U(VI) bioremediation is stimulated by the addition of an organic electron donor such as acetate (Anderson et al., 2003; Brodie et al., 2006; Chandler et al., 2006; Cardenas et al., 2008), and when Fe(III) is the primary electron acceptor supporting the growth of *Geobacter* species under these conditions (Finneran, 2002). *Geobacter* species can transfer anaerobically electrons to the soluble U(VI) in the groundwater and reduce it to the insoluble form, U(IV). The ability of *Geobacter* to precipitate the uranium and prevent it from further spreading highlighted the importance of dissimilatory metal reduction by *Geobacter* species as a natural and promising process to limit the spread of contaminant metals and radionuclides in the subsurface environment.

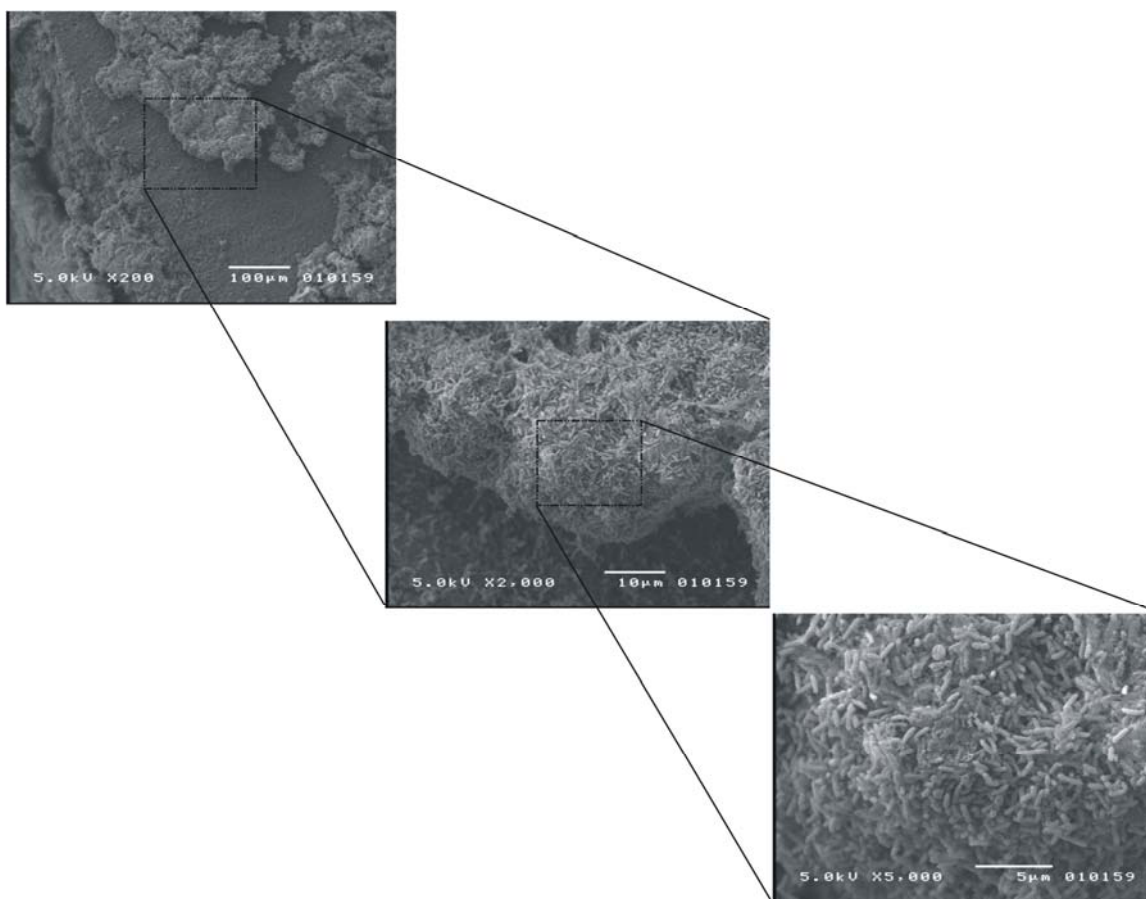


**Figure 2.** Light microscopic image of *Geobacter sulfurreducens* cells colonizing a Fe(III) oxide particle.



**Figure 3.** Ability of *Geobacter* species to couple oxidation of organic compounds to reduction of metals (Lovley et al., 1991).

*Geobacter* species are able to utilize graphite anodes as the sole electron acceptor in microbial fuel cells (MFC) or sediment fuel cells. Microbial fuel cells are devices in which microorganisms can oxidize organic compounds, *e.g.* sugars or fatty acids, and transfer the electrons derived from their oxidation to an electrode (anode), where they flow through a conductive wire or resistor to a cathode and react with the final electron acceptor, *e.g.* oxygen. Anode and cathode are separated by a proton- or cation-permeable membrane that allows the flow of cations to maintain charge neutrality, while preventing the electron acceptor from diffusing between the cathode and anode chambers, which would short-circuit the system. The electron flow can be harvested as electric current, and the maximum voltage of this “living battery” is determined by the redox potential difference between the anode and the cathode. *Geobacter* cells grow on graphite anode surfaces in MFCs, forming thick conductive biofilms and transferring electrons directly to the graphite anode (Figure 4). The harvested electricity is sufficient to power small electronic devices. Thus, to understand the composition of the conductive biofilm and to identify the factors affecting its structure and conductivity would provide an insight into the physiology of *Geobacter* and would help to optimize the parameters for higher power output (Logan and Regan, 2006; Lovley, 2006).



**Figure 4.** Scanning electron microscopic (SEM) images of the mature thick biofilm of *Geobacter sulfurreducens* wild type (DL1) strain grown on a poised graphite electrode. The images are courtesy of Dr. Hanno Richter.

*Geobacter sulfurreducens* was chosen as the model organism for investigation of the electron transfer mechanism(s), because of the availability of a complete genome sequence (Methe et al., 2003) and a genetic system (Coppi et al., 2001). In microbial fuel cells, *G. sulfurreducens* starts to produce detectable current *ca.* one day after attachment to the graphite anode. The power production increases as the biofilm grows and thickens. Once the maximum current is reached (current density of  $4.6 \text{ A/m}^2$ ), the biofilm maintains a steady power output as long electron donor is provided (Reguera et al., 2006; Nevin et al., 2008; Nevin et al., 2009). The bacterial biofilm directly transfers electrons to

the anode surface and does not require any soluble electron shuttle (Nevin and Lovley, 2000; Bond and Lovley, 2003; Torres et al., 2007; Srikanth et al., 2008). The mature biofilm grows to 30  $\mu\text{m}$  in thickness and covers the entire anode surface with differentiated pillar structures up to 55  $\mu\text{m}$  in height (Franks et al., 2009). The pH of the biofilm decreases from the outer surface of the pillars (pH = 7) to the inner layer facing the anode (pH = 6.1) (Franks, 2009). The pH gradient is probably due to protons released from the cells' oxidation of organic electron donor. Despite the lower pH near the anode, cells are metabolically active throughout the biofilm and only small differences in the transcription levels of some genes were detected for cells growing near the anode in comparison to those on the outer surface (Franks et al., 2009).

OmcZ, an outer membrane *c*-type cytochrome (encoded by the gene GSU2076), is one important component of the conductive biofilm and is essential for high-density current production. The expression level of the *omcZ* gene is similar in the inner and outer sections of the anode biofilm in MFCs (Franks et al., 2009). However, when comparing the transcription level of the *omcZ* gene in current-producing biofilms vs. non-current-producing biofilms grown on open-circuit graphite electrodes with fumarate as the sole electron acceptor, *omcZ* had a significantly higher transcription level in current-producing biofilms (Nevin et al., 2009). Deletion of the *omcZ* gene decreased current production in fuel cells drastically and irreversibly (Nevin et al., 2009). Electrochemical analysis of the anode biofilm indicated that the *G. sulfurreducens* wildtype biofilm forms a conductive network in which OmcZ functions as a bound mediator transferring electrons within the bulk of the biofilm (Richter, 2009).



Two other *c*-type cytochromes, OmcB and OmcE (encoded by genes GSU2737 and GSU0618, respectively), were also found to exhibit a higher transcription level in current-producing biofilms in comparison to those in non-current-producing graphite electrodes. However, mutant strains lacking either *omcB* or *omcE* were still able to produce maximum currents comparable to wildtype (Nevin et al., 2009). Interestingly, the  $\Delta omcB$  strain exhibited a positive redox potential in the anode biofilm, suggesting a mediator role for OmcB in electron transfer (ET) across the biofilm/electrode interface (Richter, 2009).

The gene that exhibited the greatest increase in transcription in cells grown with the electrode as the electron acceptor *versus* biofilms growing on the same surface material, but using fumarate as the electron acceptor, was *pilA*, the gene encoding the structural protein subunit of the type IV pili of *G. sulfurreducens* (Nevin et al., 2009).

#### **Type IV pili of *Geobacter sulfurreducens*: Involvement in current production and Fe(III) reduction**

*Geobacter sulfurreducens* produces type IV pilin filaments composed of PilA protein (GSU1496). The type IV pili of *G. sulfurreducens* have been shown to be electrically conductive and are commonly referred to as microbial nanowires (Reguera et al., 2005). Deletion of the *pilA* gene eliminated the high-density current production capability of *G. sulfurreducens* with no adaptation to wildtype-level current production even after an extended incubation period (Reguera et al., 2006; Nevin et al., 2008; Nevin et al., 2009). PilA is required for long-range electron transfer, enabling the cells in the outer layer of the anode biofilm to transfer electrons to the graphite electrode and to remain metabolically active (Reguera et al., 2006; Franks et al., 2009). The *pilA* gene is

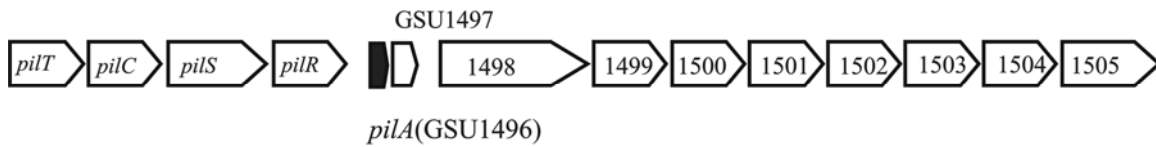
expressed at comparable levels in the outer and inner layers of the mature anode biofilm (Franks et al., 2009), which correlates with the electrochemical data suggesting that the PilA protein is an electron transfer mediator facilitating electron transfer among cells in the thick biofilm and at the cell/electrode interface (Richter, 2009).

Like the wildtype, the PilA-deficient mutant of *Geobacter sulfurreducens* is able to attach to various surfaces such as glass and Fe(III) oxide-coated glass, with fumarate as an electron acceptor, but the thicknesses of the biofilms are approximately half those of the wildtype (Reguera et al., 2007). This suggests that the pili might also play an important role within the biofilm structure by mediating cell-cell interaction as well as surface colonization.

*G. sulfurreducens* can transfer electrons to insoluble metal oxide, and type IV pili are essential for the metal-reduction process. PilA-deficient cells can still bind to iron particles, but they are incapable of growth unless a soluble electron acceptor is provided (Reguera et al., 2005; Reguera et al., 2007). Type IV pili are usually associated with the Fe(III) oxide particles and are believed to be the final electric conduit for electron transfer to Fe(III) oxide (Reguera et al., 2005).

Despite the central role of type IV pili in long-range electron transfer to Fe(III) oxide and in current production, the mechanism by which electrons are transferred through the nanowires is not fully understood. Furthermore, despite the fact that the *Geobacter sulfurreducens* gene (*pilA*) has been identified, little is known about the functional start codon of the *pilA* gene or the amino acid sequence and secondary structure of the mature protein.

The *pilA* gene of *G. sulfurreducens* is predicted to be in an operon with one other small gene on its 3' side that encodes the hypothetical protein GSU1497 ((Krushkal et al., 2010), Figure 5). The gene cluster on the 5' side of *pilA* contains *pilC*, a gene encoding one of the core proteins of pilus biogenesis, and the two-component signaling system genes *pilS* and *pilR* (Figure 5). PilR is a sigma factor known to regulate the transcription of the *pilA* gene (Juarez et al., 2009). Eight genes on the 3' side of the *pilA* operon (GSU1498 to GSU1505) are predicted to form one operon encoding a membrane protein, ATP-dependent transporters, a cell wall glycosylation enzyme, and other proteins of unknown function ((Krushkal et al., 2010), Figure 5).



**Figure 5.** Genomic organization of the pilus biogenesis genes and the 3' side genes surrounding the *pilA* gene of *Geobacter sulfurreducens*. The *pilA* gene is depicted in black.

A study of the factors regulating transcription of *pilA* indicated that transcription is initiated at two positions with two predicted ribosome-binding sites and two potential translation start codons (TTG and ATG), but did not investigate whether each of the *pilA* transcripts produced a different isoform of the PilA preprotein (Juarez et al., 2009).

Comparisons of the predicted sequence of mature *G. sulfurreducens* PilA to other type IV pilin proteins indicate that the mature *G. sulfurreducens* PilA has the highly conserved hydrophobic region known to form the core of the filamentous structure, the phenylalanine (F1) and the glutamic acid (E5) believed to be essential for the pilin

subunits' electrostatic attraction, and a hypervariable C-terminal segment. The amino acid in position 63 (of the preprotein) is serine in *G. sulfurreducens* PilA, which is found in almost all type IV pilins. However, no cysteines were detected in the PilA sequence, eliminating the possibility of disulfide bonds, which are observed in the type IV pili proteins isolated from some other bacteria (Mattick, 2002; Craig et al., 2004).

The goals of this research were to identify

1. amino acids in the mature PilA protein that are essential for reduction of insoluble Fe(III) oxide.
2. the functional start codon of the *pilA* gene, the signal peptide cleavage site and the N-terminal sequence of the mature secreted PilA protein.

The data presented in the following chapters reveal the presence of two functional translation start codons of the *pilA* gene responsible for the production of two prepilin isoforms, long and short. The two isoforms get cleaved at the conserved glycine, -1, resulting in the same mature protein sequence. The mature PilA is posttranslationally modified with a glycerolphosphate molecule at a nonconserved tyrosine (position 32) in the C-terminal segment. Point mutation of Tyr32 into phenylalanine inhibited biofilm formation on graphite surfaces and cell growth on Fe(III) oxide.

## CHAPTER 2

### THE ROLE OF TYROSINE-32 OF GEOPILIN IN BIOFILMS FORMATION AND GROWTH ON FE(III) OXIDE

#### Introduction

Long-range electron transfer (ET) by proteins is of great interest for its importance in biological reactions. Two models provide explanations for this phenomenon. According to the superexchange model (tunneling reaction), in which electrons move in a single step from the electron donor to the electron acceptor (Marcus, 1993), the electron movement is distance-dependent; electrons can typically transfer across distances of 10-14 Å (Page et al., 2003; Malak et al., 2004). The multistep electron hopping model, on the other hand, suggests that electrons hop through a chain of amino acids till they reach the electron acceptor (Jortner et al., 1998; Malak et al., 2004). This mechanism enables electrons to travel longer distances but usually requires intermediate aromatic amino acids (which have oxidizable side chains) to harbor the moving electrons for a short time (Cordes et al., 2008).

*Geobacter sulfurreducens* is a member of the *Geobacteraceae* family, which is composed of dissimilatory metal-reducing microorganisms occurring in subsurface environments. The members of this family can couple the oxidation to carbon dioxide of a wide range of organic substrates, such as acetate and toxic aromatic compounds (Lovley, 1989; Lovley et al., 1993), to the reduction of metal oxides (e.g. Fe(III), Mn(IV) and U(VI)) via anaerobic respiration. The reduction of harmful soluble metal ions to immobilized and insoluble forms gave *Geobacter* species their prominence in bioremediation of contaminated subsurface environments (Holmes et al., 2002; Anderson

et al., 2003; Holmes et al., 2007; Sanford et al., 2007). In *G. sulfurreducens*, electrons derived from central metabolism are transferred to the outside of the cells and onto Fe(III) oxide present in the environment. This process involves a number of *c*-type cytochromes in addition to the microbial nanowires (Leang et al., 2003; Mehta et al., 2005; Reguera et al., 2005; Nevin et al., 2009). The nanowires of *G. sulfurreducens* are thin appendages covering the cell surface and consist of thousands of repeats of mainly one protein subunit designated PilA. They have been reported to be electrically conductive and are considered as the final electron conduit to insoluble Fe(III) oxide in addition to being an important structural element of the current producing biofilm (Reguera et al., 2005; Reguera et al., 2006; Nevin et al., 2009; Richter, 2009). The mechanism by which electrons can be transferred through the nanowires is not fully understood. However, the fact that type IV pili can extend up to several micrometers in length from the cell suggests that the electron hopping model best describes how electrons are transferred through the nanowires to the insoluble electron acceptors. The PilA protein of *G. sulfurreducens* shares conserved amino acids with other type IV pilins. This includes the conserved phenylalanine (position 1) and glutamic acid (position 5) that are known to be essential for the electrostatic attraction among pilin subunits (Craig et al., 2006). In addition, PilA has the highly conserved hydrophobic region that folds into a helical structure in other pilins and is responsible for forming the core of the pilus (Craig et al., 2003; Craig et al., 2006). The C-terminal portion of type IV pilins in general is mainly hydrophilic and is exposed to the external environment. The sequence of this region varies from one bacterial species to another according to the protein function, except for the highly conserved serine-63 that is glycosylated in all studied pathogens.

This polysaccharide chain is believed to provide protection against the host immune system (Voisin et al., 2007). However, this modification was not observed in PilA of *Geobacter sulfurreducens*. Instead, the mass spectrometric analysis of the mature secreted PilA protein indicated posttranslational modification of tyrosine-32, a non conserved residue near the C-terminus of the mature protein, with glycerophosphate. A detailed study of the role of tyrosine-32 and glycerophosphate modification in the PilA function is presented.

## Materials and methods

### Bacterial strains and plasmids

The plasmids and bacterial strains of *Geobacter sulfurreducens* used in this study are listed in Table 1. *E. coli* strain TOP10 (Invitrogen Co., Carlsbad, CA) was used for DNA manipulation and PCR product subcloning. *G. sulfurreducens* strains were routinely cultured in acetate-fumarate medium at 25°C, the pilus expression-inducing temperature (Reguera et al., 2005) under strictly anaerobic conditions as previously described (Lovley and Phillips, 1988; Coppi et al., 2001).

Designation	Genotype or description	Source or reference
<b>Strains</b>		
DL1	Wildtype <i>Geobacter sulfurreducens</i> strain	(Caccavo et al., 1994)
DL100	DL1, intact <i>pilA</i> ; Kan <sup>r</sup>	This work
<i>pilA</i> 3(Y32F)	DL1, <i>pilA</i> contains the Y32F point mutation; Kan <sup>r</sup>	This work
$\Delta(pilA)2$	DL1, the <i>pilA</i> in-frame deletion mutant; Kan <sup>r</sup>	This work
<b>Plasmids</b>		
pLC1	An expression vector. A derivative of pRG5 (Kim et al., 2005) without the <i>tacUV5</i> & <i>lac</i> promoters; Spec <sup>r</sup>	This work
pCD341	An expression vector and source of the kanamycin resistance cassette; Kan <sup>r</sup>	(Morales et al., 1991)
pLC3	A plasmid derived from pLC1 with the last 200 bp of GSU1495, the P1 and P2 promoters, and the <i>pilA</i> gene. Cloning vector is pLC1; Spec <sup>r</sup>	This work
pLC9	<i>pilA</i> gene contains the Y32F point mutation. Mother plasmid is pLC3; Spec <sup>r</sup>	This work

**Table 1.** *Geobacter sulfurreducens* strains and plasmids used in Chapter 2



## **DNA manipulation and plasmid construction**

Total *Geobacter sulfurreducens* genomic DNA was purified using the MasterPure Complete DNA Purification Kit (Epicentre Technologies, Madison, WI). Plasmid DNA purification, PCR product purification and gel extraction were performed using the Mini Plasmid Purification, the PCR Purification and the Qiaquick Gel Extraction Kits respectively (Qiagen Inc., Valencia, CA). Restriction enzymes, T4 DNA ligase and Klenow fragment were purchased from New England Biolabs Inc., Beverly, MA. Primers for amplification of DNA fragments from the *Geobacter sulfurreducens* chromosome and for site-directed mutagenesis were purchased from Operon Biotechnologies Inc., Huntsville, AL. PCR DNA amplification reactions were performed using the high-fidelity Phusion polymerase, and the reaction conditions were set according to the manufacturer's instructions (Finnzymes Inc., Woburn MA).

The pLC1 plasmid was generated from the pRG5 expression vector (Kim et al., 2005) by removal of the *tacUV5* & *lac* promoters. Double digestion of the pRG5 vector was performed at the *Nco* I and *Ase* I restriction sites followed by treatment with the Klenow fragment for blunt ends. The digested vector was gel purified, subjected to ethanol precipitation and a 70% ethanol wash, and finally resuspended in 0.5X TE buffer (0.5 mM Tris HCl [pH 8.0], 0.5 mM EDTA) to reduce the salt content. Ligation with T4 DNA ligase followed. The new plasmid, pLC1, was transformed into *E. coli* TOP10 chemically competent cells and colonies were selected for growth on spectinomycin-containing plates. Miniprep plasmid purification was performed and the identity of pLC1 was verified by sequencing. The replication of pLC1 in *E. coli* was tested and found to have a copy number comparable to pRG5 under identical conditions. Also, no change in

growth rate or cell yield was observed with the wildtype *G. sulfurreducens* harboring pLC1 compared to wildtype without plasmid (Appendix A).

To construct the pLC3 plasmid, an insert encompassing the last 200 base pairs of the GSU1495 gene on the 5' side of *pilA*, the region including the native promoters of *pilA*, and the entire *pilA* gene was amplified by PCR using the genomic DNA of *G. sulfurreducens* as a template. The primers (rLC41F and rLC42R) introduced *Bam*H I and *Fsp* I restriction sites at the 5' and 3' ends, respectively (Table 2). The PCR product and the vector pLC1 were doubly digested at *Bam*H I and *Fsp* I restriction sites, followed by ligation and transformation into *E. coli* TOP10 chemically competent cells. The resulting plasmid was verified by sequencing to contain the entire *pilA* gene with its native promoters, and was designated pLC3.

Standard site-directed mutagenesis (QuickChange II XL Site-Directed Mutagenesis Kit, Stratagene Inc., La Jolla, CA) was performed on the pLC3 plasmid to introduce a point mutation in tyrosine-32 of the *pilA* gene, replacing the tyrosine codon (TAC) with a phenylalanine codon (TTC). The primers (the Y32F\_Fwd primer and the complementary primer Y32F\_Rev) were designed to introduce a *Bsm* I restriction site polymorphism with the mutation (Table 2). Plasmids that tested positive for single digestion at the *Bsm* I restriction site were selected and further verified by sequencing. The resulting plasmid, pLC9, contained the *pilA* native promoters and the entire *pilA* gene with a single point mutation of Tyr32 (Y32F), and was used to introduce the mutation into the chromosomal copy of the *pilA* gene as described subsequently.

Purpose	Primers and sequence	Description
<b>Strains</b>		
$\Delta(pilA)2$	<b>rLC43F:</b> 5'-AGAGCAGGTGAAGGAAGGGAGTTT-3'	To amplify fragment 1: the last 500 bp of GSU1495 ( <i>pilR</i> ).
	<b>rLC44R:</b> 5'-TCACTCCTCATCCATGCCAAATTTGCCA GGC-3'	
	<b>rLC45F:</b> 5'- <span style="border: 1px solid black;">GCCTGGCAAAATTTGGCATGGATGAGGA</span> <span style="border: 1px solid black;">GTGA</span> CTGACGGAACAGCGGGAAGTCCA GC-3'	To amplify fragment 2: the kanamycin resistance cassette. The bases that anneal to fragments 1 and 3 are boxed.
	<b>rLC46R:</b> 5'- <span style="border: 1px solid black;">TGACGATTTTCGTCACCTGGCTCCTCT</span> TGG ATCCCCCGGGCTGCAGGAATTTCG-3'	
	<b>rLC47F:</b> 5'- <span style="border: 1px solid black;">CGAATTCCTGCAGCCCGGGGATCCA</span> AG AGGAGCCAGTGACGAAAATCGTCA-3'	To amplify fragment 3: the promoter region of the <i>pilA</i> gene and to introduce the deletion version of the <i>pilA</i> . The bases that anneal to fragments 2 and 4 are boxed. The <i>pilA</i> sequence is in bold.
	<b>rLC48R:</b> 5'- <span style="border: 1px solid black;">TGTCTCCTTTCTTCTTTTGCTGC</span> <b>TTGT</b> <b>GTTGGGGTATGGGGTAATTGGCCAATT</b> ATCCGTTTAATCCTTAAACGTTATCGTG-3'	
<i>pilA3</i> (Y32F)	<b>rLC49F:</b> 5'- <span style="border: 1px solid black;">GCAGCAAAAAGAAGAAAGGAGACAG</span> <b>GA</b> <b>TGATCAAACCTATCCGCCCCGAAAGTTAAT</b> TGATTAAATACATACTGGAGGAAACCAT G-3'	To amplify fragment 4: the downstream region of the <i>pilA</i> gene and to introduce the deletion version of <i>pilA</i> . The bases that anneal to fragment 3 are boxed. The <i>pilA</i> sequence is in bold.
	<b>rLC50R:</b> 5'-CTACTGCGACTTCCACTCGGTACCAGAAA AATCAG-3'	
<i>pilA3</i> (Y32F)	<b>rLC43F:</b> 5'-AGAGCAGGTGAAGGAAGGGAGTTT-3'	To amplify fragment 1: the last 500 bp of GSU1495 ( <i>pilR</i> ) with the kanamycin resistance cassette. The bases that anneal to fragment 2 are boxed.
	<b>rLC46R:</b> 5'- <span style="border: 1px solid black;">TGACGATTTTCGTCACCTGGCTCCTCT</span> TGG ATCCCCCGGGCTGCAGGAATTTCG-3'	

<b>rLC47F:</b> 5'- <u>CGAATTCCTGCAGCCCGGGGATCCA</u> AG AGGAGCCAGTGACGAAAATCGTCA-3'	To amplify fragment 2: the <i>pilA</i> gene with the Y32F mutation. The bases that anneal to fragment 1 are boxed. Reverse primer binds to bases 37 to 70 of gene GSU1497
<b>rLC51R:</b> 5'-CTATTGCGCACAATGGCTATTCCCTGCAT TGCGA-3'	

<b>Plasmids</b>		
pLC3	<b>rLC41F:</b> 5'-GATAGGATCCGTCACCGAGTGCGAACTGC C-3'  <b>rLC42R:</b> 5'-CTATTGCGCACAATGGCTATTCCCTGCAT TGCGA-3'	To construct a plasmid containing the entire <i>pilA</i> gene and its native promoters. <i>Bam</i> H I and <i>Fsp</i> I sites in italics and underlined.
pLC9	<b>Y32F_Fwd:</b> 5'-CAGTTCTCGGCGTATCGTGTCAAGGCATT CAACAGCGCGGCGTCAAGCG-3'  <b>Y32F_Rev:</b> 5'-CGCTTGACGCCGCGCTGTTGAATGCCTTG ACACGATACGCCGAGAACTG-3'	For site-directed mutagenesis of the <i>pilA</i> gene. The base pairs that were replaced are in bold. The <i>Bsm</i> I site is in italics and underlined.

**Table 2.** List of primers used in Chapter 2

### Construction of *Geobacter sulfurreducens* mutant strains

Both mutant strains used in this study, *pilA3*(Y32F) and  $\Delta(pilA)2$ , were constructed by recombinant PCR as previously described (Coppi et al., 2001). The kanamycin resistance cassette was placed in the same position in both strains, on the 5' side of the *pilA* promoter region. The isogenic control strain, DL100, which has the wildtype version of *pilA*, differs from the wildtype strain DL1 only in having the kanamycin resistance cassette inserted before the promoter region of *pilA*. Chromosomal DNA of DL100 was used as a PCR template for amplification of a fragment containing the last 500 bp of the GSU1495 gene and the kanamycin resistance cassette while

constructing the mutagenic linear fragment for *pilA3*(Y32F) (see primers rLC43F and rLC46R; Table 2). The remaining DNA fragments for construction of *pilA3*(Y32F) and  $\Delta(pilA)2$  were amplified with the corresponding primers listed in Table 2. The pLC9 plasmid was the template for amplification of the *pilA* gene with the Y32F mutation. In the case of the in-frame deletion mutant strain,  $\Delta(pilA)2$ , amplicons of the 5' and 3' flanking regions of *pilA*, including the first ten and last ten codons of the *pilA* gene, were fused by annealing of an overlapping sequence of 24 base pairs (codons 11 to 18 of *pilA*) that was present in primers rLC48R and rLC49F (Table 2). All primers were designed to introduce flanking sequences at both ends of the amplicons that were used for DNA recombination in primerless PCR cycles. The final linear DNA fragments, each consisting of the 3' end of GSU1495, the kanamycin resistance cassette, the promoter region, a mutant *pilA* allele, and a 3' flanking sequence were gel purified and then subjected to ethanol precipitation followed by a 70% ethanol wash, resuspension in 0.5X TE buffer and transformation into *G. sulfurreducens* electrocompetent cells as previously described (Coppi et al., 2001). Mutations were confirmed by PCR with high-fidelity Phusion polymerase followed by DNA sequencing. In the case of the *pilA3*(Y32F) strain, two purified and sequenced colonies were independently used in the characterization of the mutant strain.

### **Culturing conditions and growth media**

*G. sulfurreducens* was grown under anaerobic conditions (N<sub>2</sub>/CO<sub>2</sub>: 80/20) as previously described (Coppi et al., 2001). The growth temperature was 30°C except that when fumarate was the electron acceptor, cells were grown at 25°C, the pilus expression-inducing condition (Reguera et al., 2005). Acetate (15 mM) served as the electron donor

and either fumarate (40 mM), Fe(III) citrate (60 mM) or poorly crystalline Fe(III) oxide (100 mM) was the electron acceptor.

Microbial fuel cells were set up as described previously (Bond and Lovley, 2003), and cells were grown anaerobically with acetate (10 mM) as the sole electron donor and a potentiostat-working graphite electrode as the sole electron acceptor, at 25°C (Bond and Lovley, 2003). The working electrode was poised at +300 mV (*versus* an Ag/AgCl reference electrode). Once maximum current was reached in batch mode, a continuous supply of freshwater medium with 10 mM acetate was provided to the anode chamber at a flow rate of 30 mL/hr as previously described (Reguera et al., 2006).

### **Protein preparations**

PilA protein fractions were prepared from *G. sulfurreducens* cultures grown under strictly anaerobic conditions in NBAF medium, which contains fumarate as the electron acceptor and acetate as the electron donor, at 25°C. Cells were harvested in late log phase (optical density = 0.4 - 0.5, at wavelength of 600 nm) and subjected to 15 minutes centrifugation at 5,000 x g and 4°C in a Sorvall RC 5B Plus centrifuge with a F14S-6\*250 rotor (Thermo Fisher Scientific Inc., Waltham, MA). Supernatants were collected and concentrated 100-fold in a Microcon Centrifugal Filter Device (Millipore Corp., Billerica, MA) with a 5 kDa molecular weight cut-off. Further concentration was achieved using Ultrafiltration membrane tubes with a 3 kDa molecular weight cut-off (Millipore Corp., Billerica, MA) to yield a final volume of 500 µl. This fraction was referred to as “supernatant fraction”. Pellets from the initial centrifugation were resuspended in 50 mM Tris-HCl buffer (pH 7.5) and sonicated for 4 min with a duty cycle of 15 s on, 20 s off (Sonic dismembrator F550; Fisher Scientific, PA). Resuspended

and sonicated pellets were then subjected to 15 min centrifugation at 5,000 x g and 4°C. The collected supernatant was referred to as “cell-associated fraction 1” whereas the pellet fraction was referred to as the “cell-associated fraction 2” (Figure 6). This procedure was designed after the observation that a substantial amount of the expressed PilA protein was detected in the “supernatant fraction” on Western blot (Appendix B). A similar cell fractionation approach was used previously for investigating the expression and secretion of PilA protein in *Myxococcus xanthus*, a relative of *Geobacter* and another member of the *Deltaproteobacteria* class (Wu et al., 1998). Western blotting was applied to detect the PilA protein using an anti-PilA antibody. Immunoreactive bands of “supernatant fraction” samples were used as evidence of PilA secretion, where those of the “whole cell fraction” were used to assess total PilA expression.

The PilA protein recovered in the “supernatant fraction” is expected to assemble into filaments structure. Type IV pilin monomers are cleaved at the inner membrane and assemble into pilus fibers in the periplasmic space via an electrostatic attraction among pilin subunits (Wolfgang et al., 2000; Craig et al., 2006). The growing filament crosses the outer membrane through a hole in a multimeric outer membrane protein called secretin, PilQ (Wolfgang et al., 2000; Collins et al., 2005). Taking into consideration that the mature PilA of *G. sulfurreducens* carries all the molecular elements required for assembly in homologs type IV pilins, including phenylalanine-1 and glutamate-5 (F1 and E5) responsible for subunits electrostatic attraction and the N-terminal hydrophobic segment believed to form the core of the filament, we expected that the mature PilA subunits would assemble into filaments via hydrophobic forces, which could be disrupted upon boiling and SDS treatment, as reported for homologs of pilA found in the type IV

pili of several other bacteria (Wall et al., 1998; Collins et al., 2005; Li et al., 2005; Paranjpye and Strom, 2005; Voisin et al., 2007).

Given the fact that the PilA collected in the “supernatant fraction” runs at 7kDa on Western blot and that a 7.79 kDa protein molecular weight was determined by mass spectrometric analysis, we concluded the extracellular PilA protein detected in our preparations was intact protein. Except for the soluble pilin of *N. gonorrhoeae*, an N-terminally truncated form of pilin protein, all mature type IV pilins studied have been shown to assemble into filaments (Rytönen et al., 2001).

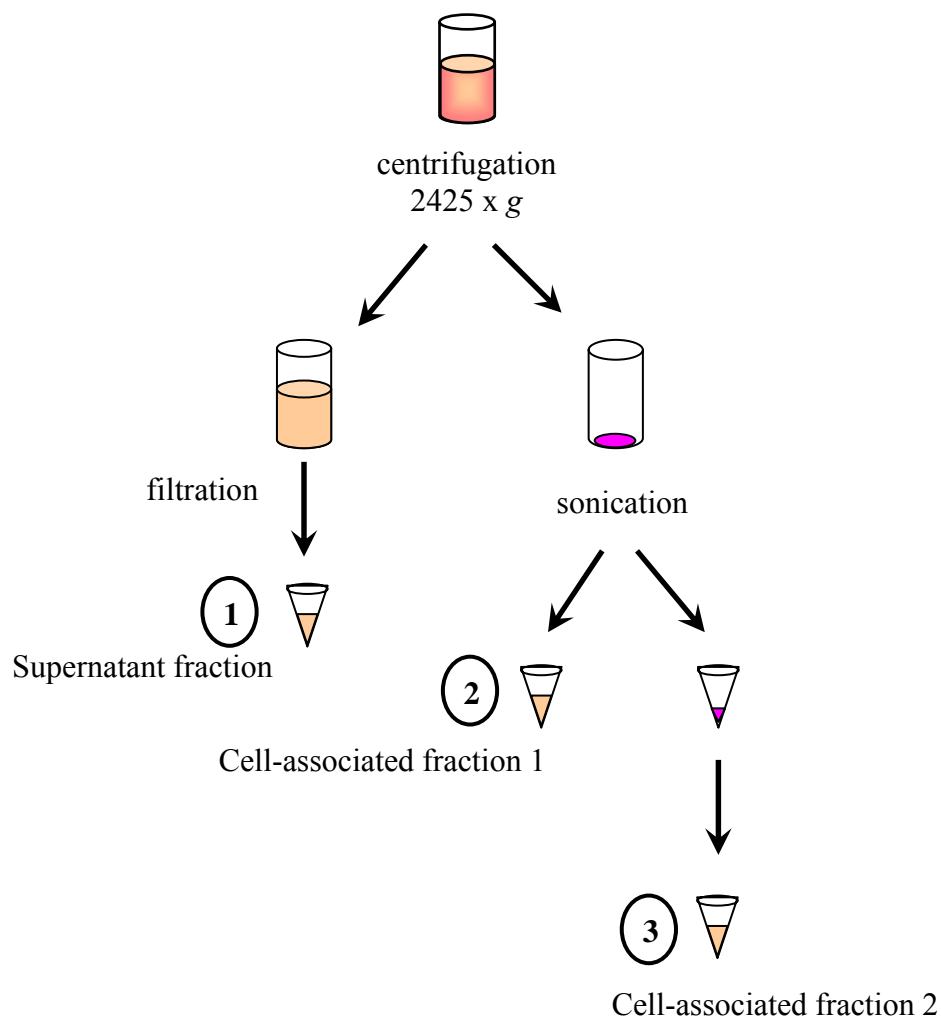
To address the possibility of intracellular PilA protein released due to cell membrane processing, the same protein preparation procedure was applied on various *pilA* mutant strains: the cleavage site mutation *pilA6*(G20L), the long isoform translation start codon mutation *pilA4*(M1) and the genetically complemented *pilA* in-frame deletion strains (see Chapters 3 and 4 for details). The failure to detect PilA in the “supernatant fraction” in those strains and the fact that PilA protein was detected in the cell-associated fractions 1 and 2, suggest that the presence of extracellular PilA is not related to the process of cell lysing, but rather to the controlled secretion of the PilA protein, as proposed in the published mechanism (Craig et al., 2006). Moreover, it was deemed unlikely for fresh a bacterial culture harvested at late log to contain substantial amount of cell lysate. In general, cell lysis occurs when cells are subjected to an external force, such as mechanical (sheering/ sonication), thermal (boiling) or chemical (SDS treatment) forces. The studied samples were fresh bacterial culture and were not subjected to any external forces before collecting the “supernatant fraction”.



For mass spectrometric analysis, the secreted PilA protein fraction prepared from DL1 wildtype strain as described above was separated by electrophoresis in a 15% Tris-tricine gel and the PilA protein band corresponding to 7 kDa was excised. The “in-gel” sample digestion, purification, and matrix-assisted laser desorption ionization tandem mass spectrometric (MALDI MS/MS) analysis were carried out at the University of Massachusetts Medical School, Laboratory for Mass Spectrometry, Worcester, MA. (University of Massachusetts Medical School).

For analysis of loosely bound outer surface *c*-type cytochromes, protein samples were prepared as previously described (Mehta et al., 2005).

All protein concentrations were determined by the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Rockford, IL) with bovine serum albumin as a standard (Smith et al., 1985).



**Figure 6.** Method of PilA protein sample fractionation and preparation. The 100 mL bacterial culture was centrifuged and the supernatant was collected and concentrated 100-fold (sample 1, supernatant fraction). Pelleted cells were resuspended, sonicated, and then centrifuged again. The supernatant was collected (sample 2, cell-associated fraction 1), and the pellet was resuspended to yield sample 3 (cell-associated fraction 2).

### **Gel electrophoresis, Western blots and heme staining analyses**

Protein samples were boiled at 100°C in SDS loading buffer (0.1 M Tris-HCl pH 6.8, 24% glycerol, 8% SDS, 0.2 M DTT and 0.02% Coomassie brilliant blue G-250) for 5 minutes, and then separated by electrophoresis in a 15% Tris-tricine gel (Ausubel, 1999) for detection of the wildtype PilA protein. Western blot analyses were performed by transferring the proteins to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA) using a semi-dry transfer unit (Trans-Blot SD, Bio-Rad Labs). The membranes were probed with a PilA-specific antibody (Yi et al., 2009) and the immunoreactive bands were visualized with the One-Step Western Kit (Genscript Corp., Piscataway, NJ) used according to the manufacturer's instructions.

The PilA antiserum was generated by Yi *et al.* (2009) against a peptide sequence corresponding to the PilA C-terminus (AIPQFSAYRVKAYNSAASSDLRN-LKTALESAFADDQTYPPEs). The peptide was over-expressed in *E. coli* as a fusion protein with maltose-binding protein and a 6xHis-tag. The cleaved peptide was used as the antigen in rabbit. The PilA polyclonal antibody was purified from the antiserum using the Melon<sup>TM</sup> Gel IgG Spin Purification Kit purchased from Pierce, Rockford IL.

Samples of loosely bound outer surface cytochromes were boiled at 100°C in a non-reducing SDS sample buffer (0.1 M Tris-HCl pH 6.8, 24% glycerol, 8% SDS and 0.02% Coomassie brilliant blue G-250), loaded on a 12% Next gel (Amersco Inc., Solon, OH) and separated by electrophoresis. The presence of the heme-binding proteins was detected by staining the gel with *N,N,N',N'*-tetramethylbenzidine as previously described (Thomas et al., 1976; Francis and Becker, 1984).

SeeBlue Plus 2 Prestained Standard (Invitrogen Corp., Carlsbad, CA) was used as the molecular weight marker for all electrophoresis gels.

### **Biofilm characterization/analysis**

Cells were stained with a nucleic acid stain, Syto 9 L7012 Component A (Invitrogen Corp., Carlsbad, CA), and examined by confocal laser scanning microscopy (CLSM) using a Leica TCS SP5 microscope with a HCX PL APO 100X (NA 1.4) objective. CLSM images were processed using the Leica LAS AF software (Leica Microsystems GmbH, Wetzlar, Germany) to create three-dimensional representations of the biofilms and cross-sections. The biofilms' thickness and percent coverage were determined using the biofilm analysis software PHLIP (Mueller et al., 2006).

### **Attachment assays**

Cells were exposed to graphite/glass surfaces in the presence of acetate (10 mM) and fumarate (40 mM) at 25°C under anaerobic conditions for 4 days and then prepared for confocal microscopic imaging as described above.

For Fe(III) oxide attachment assays, cells were grown in freshwater medium with acetate (10 mM) in the presence of a soluble electron acceptor, 40 mM fumarate, and incubated with Fe(III) oxide-coated borosilicate cover slips, prepared as described elsewhere (van Schie and Fletcher, 1999). Six slides were prepared for each strain. Cells were incubated in the presence of soluble electron donor and acceptor for 24 hours, at which time three slides per strain were imaged with CLSM. The remaining three slides were incubated for four days more with Fe(III) oxide coating as the sole electron acceptor (fumarate was removed) and then examined by CLSM.

## Results

### **Identification of the glycerophosphate post-translational modification of tyrosine-32 of the PilA protein of *Geobacter sulfurreducens***

The mature and secreted wildtype *Geobacter sulfurreducens* PilA (GSU1496) protein sample was prepared as described in the Materials and Methods section. The concentrated supernatant fraction was separated on a 15% Tris-tricine gel and stained with GelCode Blue Stain Reagent. The band corresponding to 7 kDa was excised and analyzed by mass spectrometry (MALDI MS/MS). A list of the peptides detected is shown in Table 3.

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(K)AYNSAASSDLR(N)
(K)AYNSAASSDLR(N)+ Glycerophosphate (Y)
(R)VKAYNSAASSDLR(N)
(R)VKAYNSAASSDLR(N) + Glycerophosphate (Y)
(K)TALESAFADDQTYPPEs
(K)TALESAFADDQTYPPEs + Na:Cation (C-term)

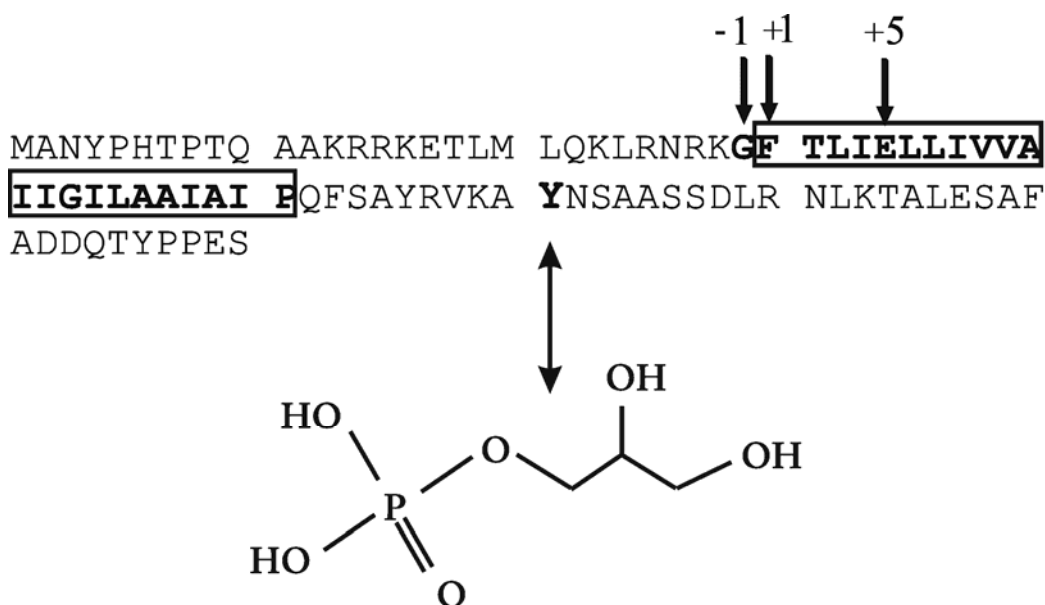
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**Table 3.** Peptides detected by MALDI MS/MS of the tryptic-digested mature and secreted PilA. The letters in parentheses indicate cleavage sites of the enzyme trypsin, which cleaves after K and R residues.

MS/MS spectra obtained from CID (Collision Induced Dissociation) and PSD (Post-Source Decay) techniques gave complete coverage of the carboxyl-terminal but not the amino-terminal sequence, presumably due to its high hydrophobicity. The three peptides in Table 3 matched those predicted for the *G. sulfurreducens* PilA sequence (Methe et al., 2003). The mass spectrometric data revealed a shift in mass in two tryptic digested peptides, (AYNSAASSDLR) and (VKAYNSAASSDLR). Both peptides appeared to be modified with a moiety of 154 Da, which was suggested to be glycerophosphate according to the protein modification database (Creasy and Cottrell, 2004; Unimod, Protein Modification for Mass Spectrometry). To verify the site of

modification, tryptic peptides were derivatized with 4-sulfophenylisothiocyanate to increase the efficiency of the PSD fragmentation (Chen et al., 2004), followed by de novo sequencing (Appendix C). Tyrosine position 32 of the mature protein was determined to be modified post-translationally with glycerophosphate (Figure 7). The hydroxyl group of tyrosine is a likely point of attachment for glycerophosphate, via either a phosphodiester bond or an ether bond. Glycerophosphate modification was first reported in a homolog type IV pilin of *N. meningitides* C311; in which the hydroxyl group of serine position 93 in the C-terminal domain was shown to be attached to the phosphate group of a glycerophosphate moiety (Stimson et al., 1996).

The amino acid sequence of *G. sulfurreducens* PilA was aligned with type IV pilin sequences of Gram-negative bacteria using ClustalW version 2 (Appendix D). Sequence analysis indicated that *G. sulfurreducens* PilA has the conserved cleavage site, glycine -1 (Strom and Lory, 1987, 1991). The mature cleaved protein contains the highly conserved hydrophobic region believed to form the core of the filamentous pilus structure (Craig et al., 2003; Craig et al., 2006). Phenylalanine (position 1) and glutamic acid (position 5) of the mature protein are the two key amino acids known to be responsible for the electrostatic attraction between pilin subunits (Craig et al., 2006). Tyrosine-32 is a non-conserved amino acid and is part of the C-terminal region of the PilA protein that is probably surface exposed (Figure 7).



**Figure 7.** Amino acid sequence of the PilA protein (GSU1496). The conserved cleavage site is glycine at position -1, so that the mature protein starts with phenylalanine at position +1. The conserved hydrophobic segment of the mature protein is enclosed in a box. Tyrosine-32 is depicted in bold and the glycerophosphate moiety is shown below it.

### Characterization of the *pilA3*(Y32F) mutant strain

To understand whether tyrosine-32 has a role in long-range electron transfer through the nanowires/pili, site-directed mutagenesis was performed to substitute tyrosine-32 with phenylalanine. The side-chain of phenylalanine is identical to that of tyrosine except it lacks the hydroxyl group to which glycerophosphate is most likely to attach. The mutation was constructed as described in the Materials and Methods section and was introduced into the chromosomal copy of the *pilA* gene via an established method using homologous recombination (Coppi et al., 2001). The point mutation was confirmed by sequencing and the amino acid replacement in the PilA protein was confirmed by mass spectrometry (*data not shown*). The resulting strain, *pilA3*(Y32F), was tested for growth on soluble electron acceptors, fumarate and Fe(III) citrate, and

appeared to have no growth phenotype (Figures 8A and 8B). The Y32F point mutation did not affect expression or secretion of the PilA protein at 25°C. All three fractions of the mutant protein, PilAY32F, namely the supernatant fraction, and cell-associated fractions 1 and 2 were observed by Western blot at similar levels to those of the wildtype DL1<sup>\*</sup> (Figure 8C). The major outer surface *c*-type cytochromes required for optimal extracellular electron transfer, OmcZ and OmcS (Mehta et al., 2005; Nevin et al., 2009), appeared to be properly localized to the outer surface, as demonstrated by the heme-stained SDS-PAGE of the loosely bound outer surface cytochrome preparation (Figure 8D).

OmcZ and OmcS were reported to be mislocalized in the *pilA* insertion mutant used in previous studies<sup>†</sup> according to heme-stained gels of loosely bound outer surface cytochrome preparations, despite the fact that the genes for these proteins were transcribed and the proteins were produced. The results of heme-stained SDS-PAGE analysis of Omc localization suggested that both cytochromes were trapped in the periplasmic region, which can be interpreted as an additional role for the PilA protein in secretion of the outer surface cytochromes. Therefore, the same approach was conducted to localize the OmcS and OmcZ cytochromes in the *pilA3*(Y32F).

Follow up studies conducted after the completion of this work on the outer membrane cytochromes expression in the *pilA3*(Y32F) using OmcS and OmcB specific antibodies on whole cells extracts sample preparation, indicated the absence of OmcS and OmcB (Dr. P. Zhou, *personal communication*). OmcB is partly embedded in the outer membrane protein (Qian et al., 2007). A strain, in which the *omcB* gene was disrupted,

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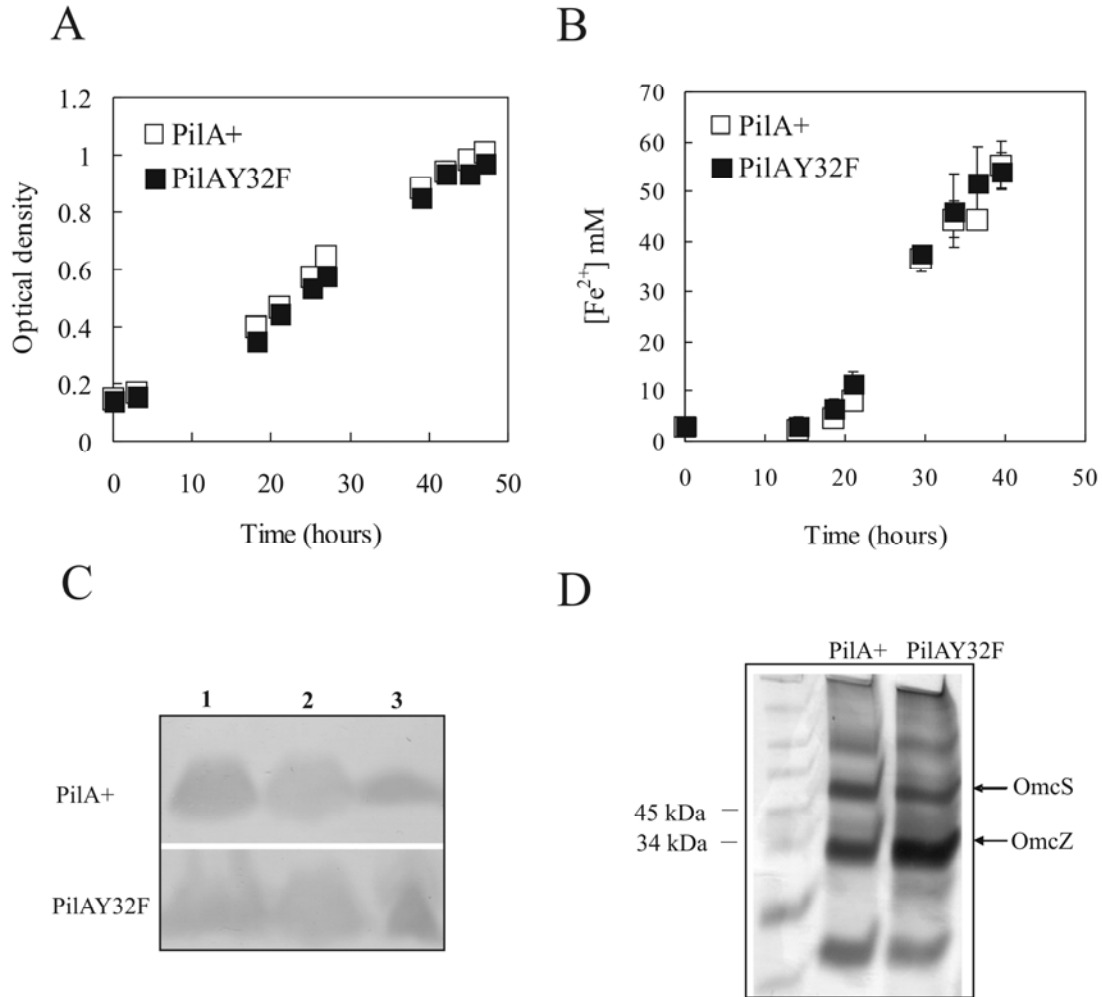
<sup>\*</sup> The intensity of the PilAY32F bands was less than those of the PilA wildtype in whole cells extracts isolated from cultures of DL100 grown at 30°C (Dr. P. Zhou, *personal communication*).

<sup>†</sup> Izallalen and Lovley- Unpublished observations.



seemed to be capable of current production and biofilm formation in microbial fuel cells (Nevin et al., 2009; Richter, 2009), but an *omcB* mutant strain was impaired in its ability to grow on Fe (III) oxide (Leang et al., 2003). No data are yet available on the localization or expression of OmcB in the *pilA* deficient mutant strain.

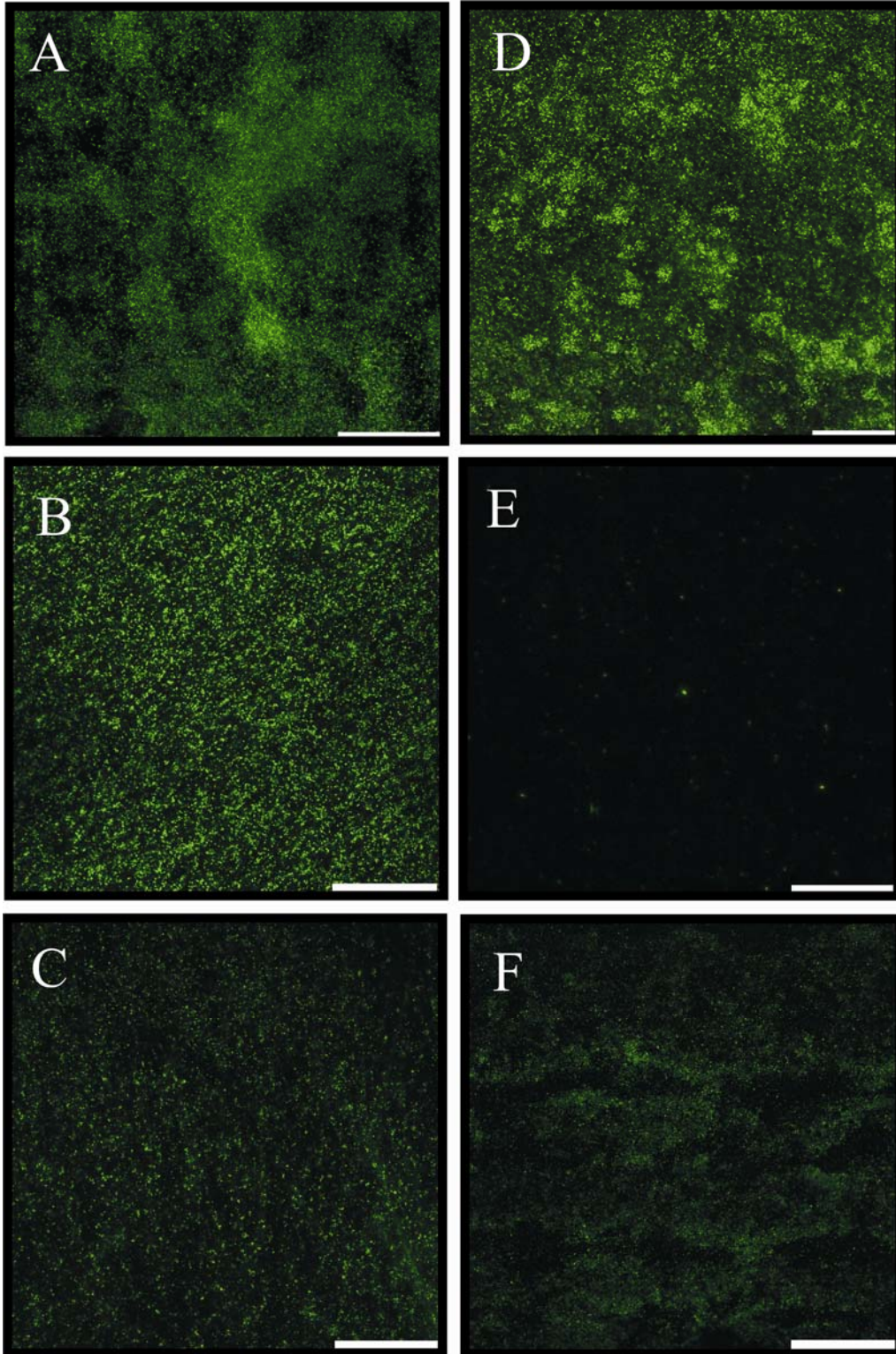
Investigations are ongoing to localize the OmcB and OmcS cytochromes and to evaluate the gene expression levels in the *pilA3*(Y32F) strain (Dr. P. Zhou, *personal communication*).



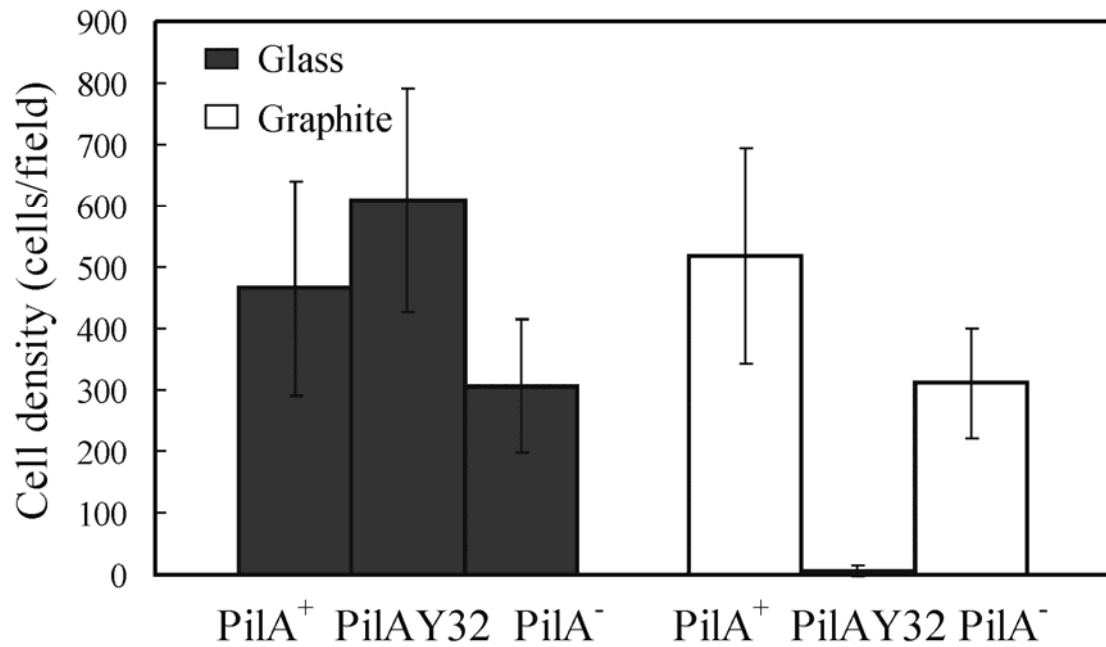
**Figure 8.** Characterization of the *pilA3*(Y32F) mutant strain. **(A)** Growth curve of the wildtype DL1 (PilA<sup>+</sup>) and the *pilA3*(Y32F) mutant (PilAY32F). Cells were grown under pilus expressing conditions (NBAF medium, 25°C). The optical densities (600 nm) are averages of six biological replicates from two independent experiments. Error bars indicate standard deviation. **(B)** Reduction of soluble Fe(III). The wildtype DL1 (PilA<sup>+</sup>) and the *pilA3*(Y32F) mutant (PilAY32F) cells were grown in freshwater medium with acetate and ferric citrate under strictly anaerobic conditions. The Fe(II) concentrations are averages of six biological replicates from two independent experiments. Error bars are standard deviations. **(C)** Western blot analysis of the wildtype DL1 (PilA<sup>+</sup>) and the *pilA3*(Y32F) mutant (PilAY32F) strains using a PilA-specific antibody. Cells were grown at 25°C. PilA fractions: (1) supernatant fraction, (2) cell-associated fraction 1 and (3) cell-associated fraction 2. All PilA protein fractions migrated at 7 kDa. Each lane contains 10 μg of total protein. **(D)** Heme-stained SDS-PAGE of loosely bound outer surface *c*-type cytochrome preparations of the wildtype DL1 (PilA<sup>+</sup>) and the *pilA3*(Y32F) mutant (PilAY32F) strains. Each lane contains 3 μg of total protein.

## Effects of the Y32F point mutation of PilA on *Geobacter sulfurreducens* cell adhesion to various surfaces

Type IV pili are known to be a structural biofilm component that mediates cell-cell interaction as well as surface colonization. In *Geobacter sulfurreducens* it is established that type IV pili are essential for maximum biomass even on surfaces that do not serve as an electron acceptor (Reguera et al., 2007). Thus, attachment assays were performed to evaluate the effect of the Y32F point mutation in the PilA protein, the structural subunit of the filamentous pili, on cell adhesion to glass and graphite. The *pilA* in-frame deletion mutant strain,  $\Delta(pilA)2$ , was used as a negative control. The wildtype DL100, the *pilA3*(Y32F) and the  $\Delta(pilA)2$  strains were grown in the presence of the soluble electron acceptor, fumarate, under strictly anaerobic conditions at 25°C, the pilus expression-inducing condition (Reguera et al., 2005). Confocal laser scanning microscopic (CLSM) images revealed no defect in the ability of the *pilA3*(Y32F) strain to attach to glass surfaces in comparison to the wildtype. However, this was not the case when the surface was graphite. The *pilA3*(Y32F) strain appeared to be severely inhibited in attachment to graphite surfaces (Figure 9). This attachment phenotype resulted from a single amino acid substitution (tyrosine into phenylalanine), was far more severe than the deletion of the entire gene; suggesting that having a non-sticky pili dominates the other factors (e.g. outer surface proteins) that allow type IV pili-minus cells to attach to graphite. These observations were supported by average cell density measurements of surface-attached cells. The biomass of the *pilA3*(Y32F) strain was in the range of 25-fold less than that of the wildtype on graphite surfaces (Figure 10).



**Figure 9.** Confocal laser scanning microscopic (CLSM) images of the *pilA3*(Y32F) biofilm on glass and graphite surfaces. CLSM images are biofilms of the wildtype DL100 (A and D), the *pilA3*(Y32F) (B and E) and the  $\Delta(pilA)2$  (C and F) strains formed on glass (A, B and C) and graphite (D, E and F) surfaces. The scale bar is 75  $\mu$ m.

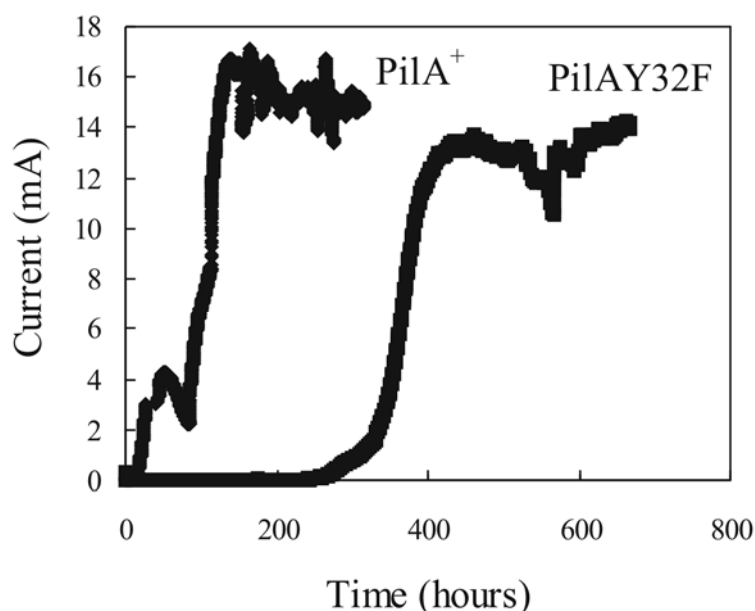


**Figure 10.** Average biomass of the *pilA3*(Y32F) mutant strain on glass and graphite surfaces. The wildtype DL100 (PilA<sup>+</sup>), the *pilA3*(Y32F) (PilAY32F) and the  $\Delta(pilA)2$  (PilA<sup>-</sup>) strains were grown anaerobically in fumarate-acetate media at 25°C on glass (solid bars) and graphite (open bars) surfaces. The results are averages of six biological replicates from two independent experiments. Error bars are standard deviations.

### **Effects of the Y32F point mutation of PilA on current production by *Geobacter sulfurreducens* in microbial fuel cells**

Based on the observations made in a scanning tunneling microscope investigation, the type IV pili of *Geobacter sulfurreducens* are believed to be conductive and serve as microbial nanowires (Reguera et al., 2005). They are essential for long-range electron transfer throughout the biofilm and to the anode surface in microbial fuel cells (Reguera et al., 2006; Nevin et al., 2008; Richter, 2009).

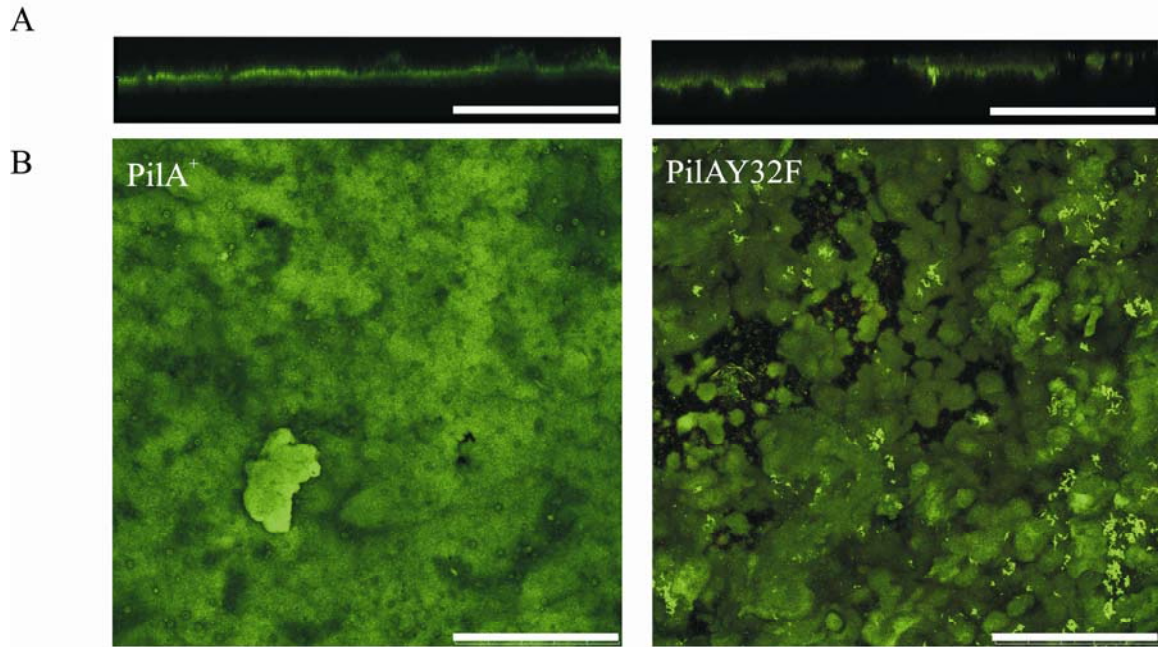
To study the impact of the Y32F mutation on generation of electricity, cells were grown on graphite electrodes in microbial fuel cells with acetate as the electron donor in strict anaerobic growth media at 25°C. The sole electron acceptor was the potentiostat-poised graphite electrode, poised at +300 mV vs. an Ag/AgCl reference electrode (Bond and Lovley, 2003). The *pilA3*(Y32F) cells exhibited a long lag phase of 200 hours before commencing to utilize the graphite electrode as electron acceptor. Once current production started the rate of increase was comparable to the wildtype strain DL100. The maximum level of current production of  $14.9 \pm 0.4$  mA was also in the same range as the maximum current produced by the wildtype (Figure 11).



**Figure 11.** Current production by the wildtype DL100 (PilA<sup>+</sup>) and the *pilA3*(Y32F) mutant (PilAY32F) strains in microbial fuel cells. Cells were grown on potentiostat-poised graphite electrodes in strictly anaerobic acetate-only media. Electrodes were poised at +300 mV (vs. an Ag/AgCl reference electrode).

Confocal laser scanning microscopic (CLSM) image stacks of the biofilms were collected after they had maintained maximum current production for 3 days in microbial fuel cells (Figure 12). A minimum of 5 random locations on each of the three biological replicates were used to determine the average biofilm thickness using the biofilm analysis software PHLIP (Mueller et al., 2006). The *pilA3*(Y32F) biofilm was comparable to the wildtype in substratum surface coverage ( $67 \pm 7$  percent coverage for the wildtype and  $52 \pm 17$  percent coverage for the mutant). The maximum pillar heights were also comparable ( $55.00 \mu\text{m}$  for the wildtype and  $50.00 \mu\text{m}$  for the mutant) with no difference in the deviation of height across the biofilm ( $0.26 \pm 0.1$  for the wildtype and  $0.27 \pm 0.1$  for the mutant (values are averages of triplicate samples  $\pm$  the standard deviations)).

Overall, no apparent structural alteration was detected in the mature biofilm of the *pilA3*(Y32F) strain.



**Figure 12.** Confocal scanning micrographs of the wildtype DL100 (PilA<sup>+</sup>) and the *pilA3*(Y32F) mutant (PilAY32F) biofilms formed on graphite anodes and producing maximum current for 3 days in microbial fuel cells. (A) Cross-sectional and (B) Top-down three-dimensional view of the biofilm. Cells were treated with a fluorescent nucleic acid-binding stain before images were obtained. Scale bars, 250  $\mu$ m.

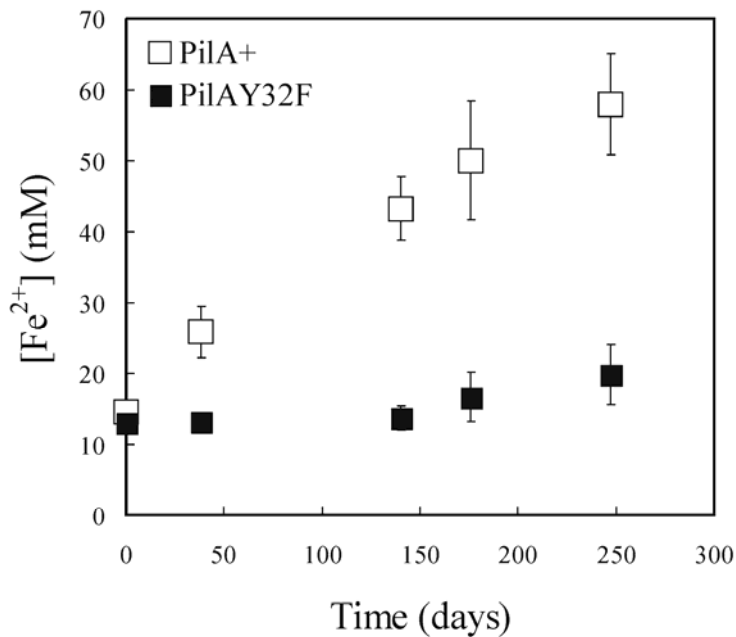
#### **Impact of the Y32F point mutation on growth of *Geobacter sulfurreducens* with insoluble iron(III) oxide.**

*Geobacter sulfurreducens* can utilize insoluble Fe(III) oxide as an electron acceptor and type IV pili are believed to be the final electric conduit for electron transfer to Fe(III) oxide (Reguera et al., 2005). Despite the fact that type IV pilus-minus cells accessed Fe(III) oxide particles and attached to Fe(III) oxide-coated surfaces, they were incapable of growth when Fe(III) oxide was the sole electron acceptor (Reguera et al.,



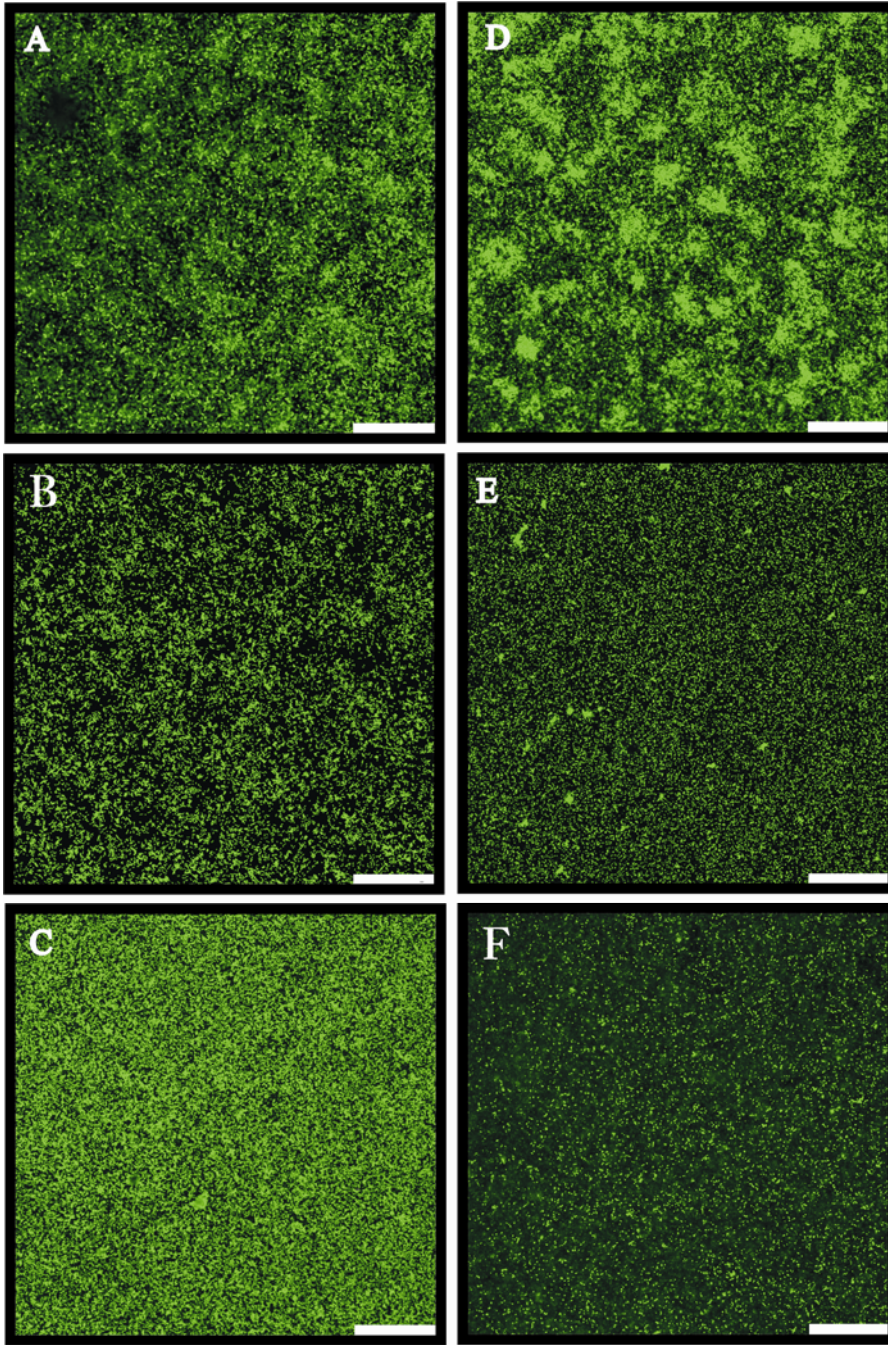
2005; Reguera et al., 2007). Therefore, it was established that type IV pili were essential for electron transfer to Fe(III) oxide, but not crucial for cell attachment.

To evaluate the contribution of tyrosine-32 and its glycerophosphate modification to cell growth on Fe(III) oxide, *pilA3*(Y32F) cells were tested for anaerobic growth with a soluble electron donor, acetate, and an insoluble electron acceptor, Fe(III) oxide (Figure 13). The electron transfer to Fe(III) oxide was monitored by measuring the concentration of Fe(II) produced. The concentration of Fe(II) measured from cultures of the wildtype DL100 gradually increased over time, whereas no significant change in Fe(II) concentration was detected for the samples taken from *pilA3*(Y32F) cultures even after six months of incubation.

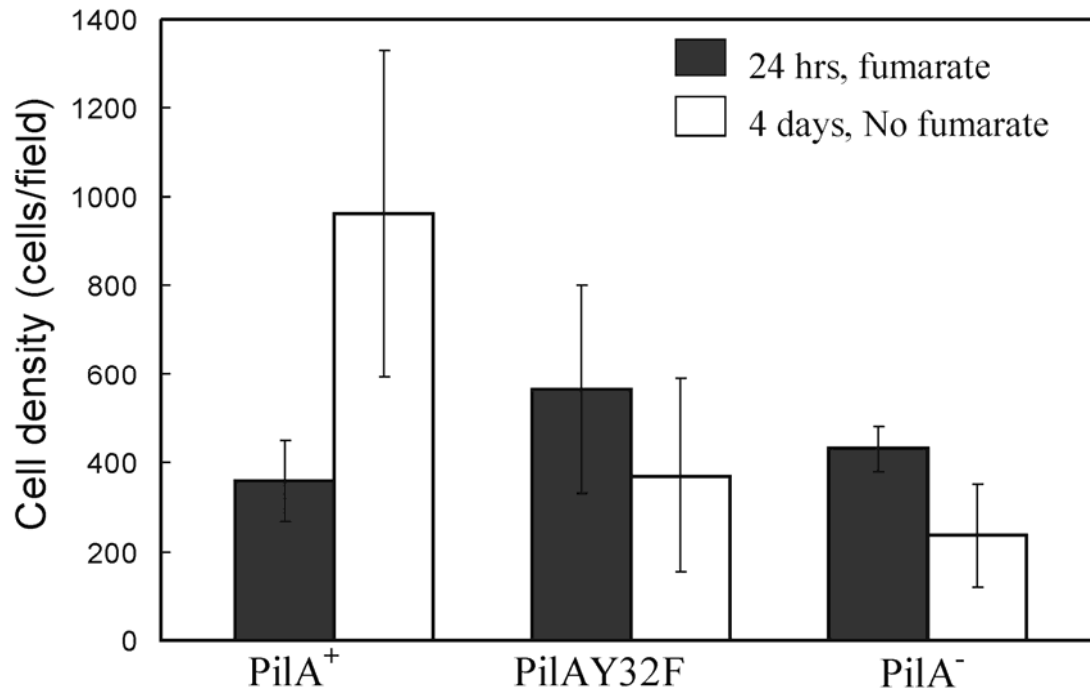


**Figure 13.** Reduction of insoluble Fe(III) oxide by the wildtype DL100 (PilA<sup>+</sup>) and the *pilA3*(Y32F) mutant (PilAY32F) strains. Cells were grown on Fe(III) oxide as the sole electron acceptor. The results are averages of eight biological replicates from two independent experiments. The error bars are standard deviations.

Attachment to Fe(III) oxide-coated glass surfaces and respiration of this electron acceptor were assayed. For all tested strains, wildtype DL100, the *pilA3*(Y32F) substitution mutant and the  $\Delta(pilA)2$  deletion mutant, cells were capable of attachment to Fe(III) oxide-coated glass surfaces after 24 hours of incubation in the presence of a soluble electron acceptor, fumarate (Figures 14A, 14B and 14C). However, after removal of fumarate and incubation of the cells for an additional 4 days with insoluble Fe(III) oxide as the sole electron acceptor, no biofilm growth was observed for the *pilA3*(Y32F) or the  $\Delta(pilA)2$  mutant strains (Figures 14E and 14F). On the contrary, visible growth of the wildtype biofilm was observed (Figure 14D). Average biomass calculations of biofilms formed on Fe(III) oxide-coated glass surfaces in the presence and the absence of soluble electron acceptor supported these observations. The analyses clearly demonstrated the increase in biomass for the wildtype biofilm and the growth deficiency of the *pilA3*(Y32F) biofilm when the sole electron acceptor was insoluble Fe(III) oxide (Figure 15).



**Figure 14.** Confocal microscopic analysis of the wildtype DL100 (A and D), *pilA3*(Y32F) (B and E) and  $\Delta$ (*pilA*)2 (C and F) biofilms formed on Fe(III) oxide-coated glass. Images were taken after incubation in the presence of fumarate for 24 hrs (A, B and C) and after removal of the fumarate and incubation of the cells without a soluble electron acceptor for 4 days (D, E and F). The scale bars are 75  $\mu$ m.



**Figure 15.** Average cell growth of the wildtype DL100 (PilA<sup>+</sup>), the *pilA3*(Y32F) (PilAY32F) and the  $\Delta(pilA)2$  (PilA<sup>-</sup>) biofilms formed on Fe(III) oxide-coated glass surfaces. Cells densities were measured after 24 hours of incubation in the presence of fumarate (solid bars) and after 4 days of incubation with no soluble electron acceptor (fumarate was removed) (open bars). The results are the average of six biological replicates from two independent experiments. Error bars are one standard deviation.

## **Discussion**

Type IV pili are common surface appendages found on most gram negative and positive bacteria. They are polymers mainly of one protein termed pilin or PilA. PilA from different bacteria are highly homologous in the N-terminal sequence, a hydrophobic segment forming the core of the growing pilus fiber. However, the C-terminus of PilA from different species varies quite a lot and is usually exposed to the outer environment. In *Geobacter sulfurreducens* type IV pili have been shown to be an important structural element in the current-producing biofilm attached to graphite electrodes and are believed to be conductive wires serving as the electron conduit to insoluble electron acceptors.

Mass spectrometric analysis of the PilA secreted by *G. sulfurreducens* confirmed the expected identity of the mature cleaved protein and indicated a posttranslational modification of a non conserved amino acid in the C-terminal domain. Tyrosine-32 of the secreted PilA was found to be modified with glycerolphosphate. To evaluate the effect of this modification and tyrosine-32 in PilA function, Tyr32 was replaced with phenylalanine and the resulting strain, *pilA3*(Y32F), was tested for biofilm formation on various surfaces. The *pilA3*(Y32F) mutant cells were deficient in attachment to graphite in the presence of soluble electron acceptor, and thus, a lag phase of 200 hours was observed before the *pilA3*(Y32F) mutant was able to use the graphite anode as electron acceptor. This attachment deficiency is a consequence of a single amino acid change; suggesting that the glycerolphosphate moiety attached to tyrosine-32 is exposed to the external environment and contributes to the pilus surface chemistry (Marceau et al., 1998; Smedley et al., 2005). Interestingly, the Y32F mutation resulted in less cell attachment to graphite than the outright deletion of the whole *pilA* gene, when soluble electron acceptor

was provided. Deletion of the *pilA* gene may have allowed other outer surface proteins to replace the pili filaments and increase the affinity of the cell outer surface towards graphite. In general, single amino acid substitutions can facilitate more systematic objective investigations of type IV pili function relative to the deletion mutant.

The mutant strain *pilA3*(Y32F) grew as well as the wildtype on soluble electron acceptor, fumarate and ferric citrate, but did not grow on insoluble Fe(III) oxide even after extended period of 6 months. The *pilA3*(Y32F) was still capable of forming a biofilm on Fe(III) coated glass, but was not able to use iron (III) oxide as an electron acceptor. The ability to grow on soluble electron acceptors, but not insoluble Fe(III) oxide, is the same phenotype reported previously for a *pilA*-minus mutant strain (Reguera et al., 2005). However, unlike the *pilA*-deficient strain, the *pilA3*(Y32F) strain could still produce and secrete PilA protein under the applied experimental conditions. The lack of growth on Fe(III) oxide of the *pilA3*(Y32F) cells could be attributed to attachment deficiency to the insoluble electron acceptor. It is established that *Geobacter sulfurreducens* does not utilize an electron shuttle (Nevin and Lovley, 2000) and a physical interaction between pili and the Fe (III) particles is required for cell growth. It is plausible that the glycerolphosphate moiety may bind Fe(III) and aid in bringing Fe(III) oxide particles close to the pilus surface, thereby facilitating Fe(III) oxide reduction. It has been reported that phosphorylation of tyrosine enhances the stoichiometry of Fe(III) binding to peptides from 1 to 2 (Baldwin et al., 2008). The glycerolphosphate moiety likely binds to the hydroxyl group of tyrosine and not to any of the carbon atoms of the aromatic ring, because the phenotypes observed for the *pilA3*(Y32F) strain were due to the removal of the hydroxyl group (substitution of tyrosine with phenylalanine).

Furthermore, mass spectrometry results indicated that phenylalanine was not modified with glycerolphosphate\*. Thus, the glycerolphosphate has a potential function in iron-binding, which would be useful for attachment and electron-transfer to Iron (III). This hypothesis is consistent with what has previously been visualized under a transmission electron microscope with Fe(III) oxide particles localized along the length of the pili (Reguera et al., 2005). Further investigations are needed to test this hypothesis.

Tyrosine-32 was not essential for long-range electron transfer to poised graphite electrodes in microbial fuel cells. Once current production was initiated, the rate of current increase and the maximum current produced were comparable to those of the wildtype. The delay in current production is attributed to the attachment deficiency to graphite surfaces, but once the first layer of biofilm is initiated on the anode, the biofilm could grow fast and electron transfer could be mediated by extracellular cytochromes. In the case of respiration with Fe(III) oxide, cells are in constant need to attach via pili to new Fe(III) oxide particle, once the previous Fe(III) particle has been reduced to soluble Fe(II). Therefore, both processes (electron transfer along pili and pili-Fe(III) binding) are equally important for cell growth in Fe(III) oxide. Another explanation is the contribution of some heme binding cytochromes such as OmcS and OmcB. OmcB and OmcS were previously reported to be important for cell growth on Fe(III) oxide (Leang et al., 2003; Mehta et al., 2005), but not for conductive biofilm formation or current production in microbial fuel cells (Nevin et al., 2009; Richter, 2009). Based on recent observations, OmcS and OmcB were not detected on Western blots of the whole cell extracts from *pilA3*(Y32F) strain (Dr. P. Zhou, *personal communication*). Investigations are being

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\* Due to high level of noise, one cannot rely heavily on this data.

conducted to evaluate the gene transcriptional level for both cytochromes and to understand the link between the Y32F mutation of PilA protein and the expression of OmcB and OmcS.

### **Summary and future directions**

The type IV pili of *Geobacter sulfurreducens* are an important element in surface colonization and in long-range electron transfer to insoluble electron acceptors. Tyrosine-32 of PilA is a key amino acid for biofilm formation and cell growth on Fe(III) oxide. Other charged or aromatic amino acids near the C-terminus of PilA (e.g. two more tyrosine residues) might also be essential for the PilA function. Site-directed mutagenesis is a feasible technique to study the role of those amino acids and to explore the mechanisms by which electrons are transferred through pili into insoluble Fe(III) oxide.



## CHAPTER 3

### DIFFERENTIATION OF THE ROLES OF TWO ISOFORMS OF GEOPILIN PREPROTEIN IN *GEOBACTER SULFURREDUCTENS*

#### Introduction

*Geobacteraceae* are anaerobic bacteria belonging to the *Deltaproteobacteria*. *Geobacter* species are Fe(III) reducers highly abundant in subsurface environments in which Fe(III) reduction is an important process to dispose of electrons derived from microbial oxidation of fermentation end products (e.g. acetate, alcohols and toxic aromatic compounds that were introduced as contaminants into the ground by human activity) (Lovley, 1989; Lovley et al., 1993; Finneran, 2002; Holmes et al., 2002; Anderson et al., 2003; North et al., 2004; Vrionis et al., 2005). *Geobacter* species can use as electron acceptors several insoluble oxide of metals such as Fe(III), Mn(IV), U(VI) and V(V) (Lovley and Phillips, 1988; Lovley, 1991a; Caccavo et al., 1994; Lovley et al., 2004; Ortiz-Bernad et al., 2004), as well as humic substances (Lovley, 1996; Voordeckers et al., 2010) and graphite anodes (Bond et al., 2002; Lovley, 2006). Investigations of the mechanism of electron transfer to insoluble electron acceptors have been mostly conducted in *Geobacter sulfurreducens* due to the availability of a complete genomic sequence (Methe et al., 2003) and a genetic system (Coppi et al., 2001). Several components of the cell have been identified as important for long-range electron transfer to Fe(III) oxide and/or to graphite anodes. These include MacA, a *c*-type cytochrome located in the inner membrane (Butler et al., 2004), and several outer membrane *c*-type cytochromes, OmcB, OmcS, OmcE and OmcZ (Leang et al., 2003; Leang and Lovley, 2005; Mehta et al., 2005; Kim et al., 2006; Nevin et al., 2009; Richter, 2009), in addition

to the extracellular appendages known as “nanowires.” The nanowires of *G. sulfurreducens* have been reported to be type IV pili and to be essential for electron transfer to Fe(III) oxide (Afkar et al., 2005), for optimal current production when a graphite anode is the sole extracellular electron acceptor (Reguera et al., 2006; Nevin et al., 2008; Richter, 2009) and for thick biofilm formation on various surfaces (Reguera et al., 2007; Nevin et al., 2009). The type IV pili of *G. sulfurreducens*, like those of other Gram-negative bacteria, are presumably polymers of one protein, pilin (PilA), encoded by the *pilA* gene (GSU1496) (Afkar et al., 2005).

Type IV pilins in Gram-negative bacteria are synthesized as prepilins with a leader sequence that is cleaved at a conserved glycine (position -1) by a specific leader peptidase, PilD, after the protein reaches the inner membrane (Strom and Lory, 1991; Arts et al., 2007; Francetic et al., 2007). The mature protein is secreted and multiple units are assembled into the pilus fiber (Mattick, 2002; Craig et al., 2006). Several genes are involved in pilus biogenesis, of which few are conserved across the Gram-negative bacteria (Hobbs and Mattick, 1993; Lauer et al., 1993; Alm and Mattick, 1997).

Transcription of type IV pilin genes is regulated by, at least, the putative two-component regulatory system PilS/PilR (Ishimoto and Lory, 1992; Hobbs and Mattick, 1993; Jin et al., 1994a; Boyd and Lory, 1996). PilS autophosphorylates in response to an environmental signal, and transfers the phosphate to the response regulator PilR. Phosphorylated PilR modulates transcription at a promoter upstream of the pilin gene. In *P. aeruginosa* and *M. xanthus*, PilR is an RpoN ( $\sigma^{54}$ )-dependent transcriptional activator that binds to a sequence upstream of the *pilA* transcription initiation site (Ishimoto and Lory, 1989; Hobbs et al., 1993; Strom and Lory, 1993; Jin et al., 1994b; Wu and Kaiser,

1997). In the case of *N. gonorrhoeae* the upstream region of *pilE* (the type IV pilin homolog) contains three promoters, of which one is a  $\sigma^{54}$ -dependent and the other two are  $\sigma^{70}$ -dependent. Depending on the expression conditions, *pilE* transcription is reported to be either  $\sigma^{54}$ - or  $\sigma^{70}$ -dependent (Taha et al., 1991; Fyfe et al., 1995).

In *G. sulfurreducens*, the *pilS* and *pilR* genes are located on the 5' side of the *pilA* gene, and PilR (GSU1495) likely functions as an RpoN-dependent enhancer-binding protein that binds to a specific sequence located in a putative promoter upstream of the *pilA* gene (Figure 16) (Juarez et al., 2009). Mapping of the 5' end of the *pilA* transcript using RNA isolated from the wildtype strain revealed the presence of a long and a short transcript of the *pilA* gene. The long transcript is RpoN-dependent and unlike the short transcript (RpoN-independent), it was not detected in RNA isolated from the *pilR* mutant strain (Juarez et al., 2009). Juárez *et al.* identified two transcription start sites and predicted two translation start codons with independent ribosomal binding sites (Figure 16), but did not investigate whether each of the *pilA* transcripts produced a different isoform of the PilA preprotein. Characterization of the PilR-deficient mutant strain revealed phenotypes similar to those found in the PilA-deficient strain. Both *pilR* and *pilA* mutant strains were deficient in growth on insoluble Fe(III) oxide and exhibited a decreased ability to attach to glass surfaces (Afkar et al., 2005; Reguera et al., 2007; Juarez et al., 2009). This suggested that the PilA isoform resulting from the long transcript (the transcript that was not detected in the *pilR* mutant strain) was necessary for growth and attachment.

The purpose of this study was to investigate the hypothesis of two functional translation start codons and the possibility that two isoforms of PilA preprotein exist, and to determine the role of each of the two PilA preprotein isoforms.



**Figure 16.** (A) Analysis of the nucleotide sequence of the *pilA* gene and the 5' region. The putative P1 and P2 promoters of the *pilA* gene are depicted in red. The sequence where PilR binds to the RpoN-dependent promoter is boxed in green. Arrows mark the two transcription initiation sites. The two ribosome-binding sites are italicized and underlined. The predicted translation start codons (TTG and ATG) are depicted in blue. The figure source is Juárez *et al.* 2009. (B) Amino acid sequence of the two prepilin proteins, long and short. The long prepilin sequence is colored in black; the short prepilin sequence is colored in blue. Both prepilin isoforms are cleaved at the same site (indicated by an arrow), leading to the single mature PilA protein. The hydrophobic segment of the mature PilA is boxed and the two residues responsible for electrostatic attraction, F1 and E5, are in larger font.

## Materials and methods

### Bacterial strains and plasmids

Strains of *G. sulfurreducens*, the wildtype and the constructed mutants, in addition to the plasmids generated in this work, are listed in Table 4. *E. coli* strain TOP10 was purchased from Invitrogen Co., Carlsbad, CA and was used for subcloning of PCR products and for DNA manipulation.

Designation	Genotype or description	Source or reference
<b>Strains</b>		
DL1	Wildtype <i>Geobacter sulfurreducens</i> strain	(Caccavo et al., 1994)
DL100	DL1, intact <i>pilA</i> ; Kan <sup>r</sup>	Chapter 2
<i>pilA4</i> (M1)	DL1, <i>pilA</i> contains a TTG→CCC mutation of the long isoform's start codon; Kan <sup>r</sup>	This work
<i>pilA5</i> (RBS)	DL1, <i>pilA</i> contains synonymous mutations of the ribosome-binding site of the short isoform; Kan <sup>r</sup>	This work
<i>pilA6</i> (G20L)	DL1, <i>pilA</i> contains a mutation at the peptidase cleavage site (glycine -1 to leucine); Kan <sup>r</sup>	This work
<i>pilA7</i>	DL1, <i>pilA</i> contains the TTG→CCC mutation of the long isoform's start codon and the ribosome-binding site mutations of the short isoform; Kan <sup>r</sup>	This work
$\Delta$ ( <i>pilA</i> )2	DL1, the <i>pilA</i> in-frame deletion mutant; Kan <sup>r</sup>	Chapter 2
<b>Plasmids</b>		
pCD341	Expression vector, and source of kanamycin resistance cassette; Kan <sup>r</sup>	(Morales et al., 1991)
pLC1	A derivative of pRG5 (Kim et al., 2005) but without the <i>tacUV5</i> & <i>lac</i> promoters; Spec <sup>r</sup>	Chapter 2
pLC3	pLC1 containing the wildtype version of the <i>pilA</i> gene; Spec <sup>r</sup>	Chapter 2

pLC8	pLC3 containing the <i>pilA</i> gene with the TTG→CCC start codon mutation; Spec <sup>r</sup>	This work
pLC13	pLC3 containing the <i>pilA</i> gene with the ribosomal binding site mutations; Spec <sup>r</sup>	This work
pLC14	pLC3 containing the <i>pilA</i> gene with the TTG→CCC start codon mutation and the ribosome-binding site mutations; Spec <sup>r</sup>	This work
pLC20	pLC3 containing the <i>pilA</i> gene with the G20L mutation (glycine -1 into leucine); Spec <sup>r</sup>	This work

**Table 4.** *Geobacter sulfurreducens* strains and plasmids used in Chapter 3

### DNA manipulations and plasmid construction

Total *G. sulfurreducens* genomic DNA was purified using the MasterPure Complete DNA Purification Kit (Epicentre Technologies, Madison, WI). Plasmid DNA purification, PCR product purification and gel extraction were performed using the QIAprep Spin Mini Plasmid Purification, QIAquick PCR Purification and QIAquick Gel Extraction Kits, respectively (QIAGEN Inc., Valencia, CA). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs Inc., Beverly, MA. Primers for PCR amplification of DNA and for site-directed mutagenesis were purchased from Operon Biotechnologies Inc., Huntsville, AL. All PCR reactions were performed using high-fidelity Phusion polymerase and the reaction conditions were set according to the manufacturer's instructions (Finnzymes Inc., Woburn MA).

Plasmid pLC3 carrying the wildtype *pilA* gene, constructed as described in Chapter 2, served as a template to introduce all the *pilA* mutations in this work using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene Inc., La Jolla, CA). All primers were designed either to introduce a restriction site polymorphism or to eliminate a naturally occurring unique site in the *pilA* gene (Table 5). These restriction sites were

used for subsequent screening to verify that the plasmid carried the desired mutations. All plasmids were checked by DNA sequencing and the *pilA* gene was confirmed to carry only the desired mutations.

Purpose	Primers and sequence	Description
<b>Strains</b>		
<i>pilA4</i> (M1)/ <i>pilA5</i> (RBS)/ <i>pilA6</i> (G20L)/ <i>pilA7</i>	<b>rLC43F:</b> 5'-AGAGCAGGTGAAGGAAGGGAGTTT-3'  <b>rLC46R:</b> 5'- <span style="border: 1px solid black;">GACGATTTTCGTCACTGGCTCCTCT</span> TGG ATCCCCGGGGCTGCAGGAATTCG-3'  <b>rLC47F:</b> 5'- <span style="border: 1px solid black;">CGAATTCCTGCAGCCCGGGGGATCCA</span> A GAGGAGCCAGTGACGAAAATCGTCA-3'  <b>rLC51R:</b> 5'-TATTGCGCACAATGGCTATTCCCTGCAT TGCGA-3'	To amplify fragment 1: the last 500 bp of GSU1495 ( <i>pilR</i> ) with the kanamycin resistance cassette. The bases that anneal to fragment 2 are enclosed in a box.  To amplify fragment 2: the <i>pilA</i> gene with various mutations. The bases that anneal to fragment 1 are in a box.
<b>Plasmids</b>		
pLC8	<b>M1_Fwd:</b> 5'-GATTAAACGGATAA <b>CCCGCCA</b> ATTACC CCCATACCCCAACACAAGC-3'  <b>M1_Rev:</b> 5'-TTGTGTTGGGGTATGGGGGTAATTGGC <b>GGGTTATCCGTTTAATCC</b> -3'	To introduce the TTG→CCC start codon mutation in the <i>pilA</i> gene. The base pairs that were replaced are depicted in bold. Introduction of this mutation disrupts the naturally occurring unique <i>Msc</i> I site in the <i>pilA</i> gene.
pLC13	<b>RBS_Fwd:</b> 5'-CCCCCATACCCCAACACAAGCAGCAAA ACGTCGTAA <b>AGAACTCTT</b> ATGCTTCAGA AACTCAG-3'  <b>RBS_Rev:</b> 5'-TGAGTTTCTGAAGCATAAG <b>AGTTTCTTT</b> <b>ACGACGTTT</b> TGCTGCTTGTGTTGGGGTATG GGGG-3'	To introduce the ribosome-binding site mutations of the short isoform. The base pairs that were replaced are depicted in bold. Introduction of these mutations disrupts the naturally occurring unique <i>BsmA</i> I site in the <i>pilA</i> gene.

pLC20	<b>G20L_Fwd:</b> 5'-CTCAGAAACAGGAAGCTTTTTCACCCTT ATCGAGCTGCTGATCG-3'  <b>G20L_Rev:</b> 5'-GATCAGCAGCTCGATAAGGGTGAAAAG CTTCCTGTTTCTGAG-3'	To introduce the peptidase cleavage site mutation in the <i>pilA</i> gene. The restriction site polymorphism introduced ( <i>Hind</i> III) is underlined.
RT-PCR	<b>pilAq_Fwd:</b> 5'-GGCCAATTACCCCCATAC-3'  <b>pilAq_Rev:</b> 5'-ACTTTCGGGCGGATAGGTTT-3'	Forward and reverse primers for RT-PCR reactions used to amplify the longer isoform of the <i>pilA</i> gene in different strains.

**Table 5.** List of primers used in Chapter 3

### Construction of *Geobacter sulfurreducens* mutant strains

A recombinant PCR protocol (Coppi et al., 2001) was used to construct all mutant strains used in this study as described in Chapter 2. Briefly, pLC3-derived plasmids carrying the *pilA* gene with the desired mutations were used as templates for amplification of the *pilA* gene, generating linear mutagenic fragments. The point mutations were introduced into the chromosomal copy of the *pilA* gene and resistance to kanamycin due to the marker inserted on the 5' side of the P1 and P2 promoters of *pilA* was used to select for recombination of each fragment with the *G. sulfurreducens* chromosome. Mutations were confirmed by PCR with high-fidelity Phusion polymerase and different sets of primers to map the *pilA* region, followed by restriction digestion to verify the introduced restriction site polymorphisms in the *pilA* gene and by sequencing.

### Qualitative reverse transcriptase polymerase chain reaction (RT-PCR)

*G. sulfurreducens* strains (mutants and wildtype) were cultured in NBAF medium (Coppi et al., 2001) at the pilus expression-inducing temperature, 25°C (Afkar et al.,



2005). Cells were harvested in mid-log phase and the total RNA was purified following the protocol of the RNeasy Midi kit (QIAGEN Inc., Valencia, CA). Isolated RNA was treated to remove DNA contamination using the DNA-free<sup>TM</sup> kit (Ambion). Complete removal of DNA was verified by a PCR reaction using the RNA sample as a template. The clean RNA sample was checked for quality on a 1% agarose gel and the total RNA concentration was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA sample was stored in small aliquots at -80°C for further use. cDNA was synthesized using the Enhanced Avian RT First Strand Synthesis kit (Sigma Aldrich St. Louis MO), using random nonamers. PCR reactions were performed using the cDNA of different strains as templates and primers designed to amplify the long transcript of the *pilA* gene (pilAq\_Fwd and pilAq\_Rev; Table 5).

### **Culturing conditions and growth media**

*G. sulfurreducens* wildtype and mutant strains were routinely cultured in NBAF medium with 15 mM acetate and 40 mM fumarate under strict anaerobic conditions (N<sub>2</sub>/CO<sub>2</sub>: 80/20) and at 25°C. Plating and incubation on solid agar media were performed as previously described (Coppi et al., 2001). Liquid cultures were streaked or plated on NBAF medium containing 1.5% agar, 0.1% yeast extract and 1 mM cysteine in an anaerobic chamber at 30°C.

In microbial fuel cells, bacterial cells were first grown in 200 mL batch cultures of freshwater medium with 10 mM acetate as the sole electron donor and the graphite anode poised at +300 mV (*versus* an Ag/AgCl reference electrode) as the sole electron

acceptor (Bond and Lovley, 2003). The system was switched to flow-through mode with 30 mL/hr of 10 mM acetate once current production was initiated (Reguera et al., 2006).

### **Western blots and heme staining analyses**

Protein samples for Western blot were prepared as described in full detail in Chapter 2 to isolate the “supernatant fraction”, “cell-associated fraction 1” and “cell-associated fraction 2” containing PilA protein. Protein samples were boiled at 100°C in SDS loading buffer (0.1 M Tris-HCl pH 6.8, 24% glycerol, 8% SDS, 0.2 M DTT and 0.02% Coomassie brilliant blue G-250) for 5 minutes, run on 15% Tris-tricine gels and separated according to molecular weight by SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA) using a semi-dry transfer unit (Trans-Blot SD, Bio-Rad Labs). The membrane was subjected to a PilA-specific polyclonal antibody (Yi et al., 2009) and the PilA bands were visualized using a One-Step Western kit (Genscript Corp., Piscataway, NJ).

The PilA antiserum used in this study was generated previously against a peptide sequence of the PilA C-terminus (AIPQFSAYRVKAYNSAASSDLRNLTAL-ESAFADDQTYPPES). The peptide was overexpressed in *E. coli* as a fusion protein with maltose binding protein containing a 6xHis-tag. The cleaved peptide was used as the antigen in rabbit. The PilA polyclonal antibody was purified from the antiserum using the Melon™ Gel IgG Spin Purification Kit purchased from Pierce, Rockford IL.

To detect the outer surface heme-bound *c*-type cytochromes, samples were prepared as described in Chapter 2 and separated by electrophoresis using 12% Next gels (Amresco Inc., Solon OH). *N,N,N',N'*-tetramethylbenzidine was used for heme staining according to the published protocols (Thomas et al., 1976; Francis and Becker, 1984).

SeeBlue Plus 2 Prestained Standard (Invitrogen Corp., Carlsbad, CA) was used for all electrophoresis gels.

### **Biofilm characterization/analysis**

All bacterial biofilms, regardless of the surface material, were treated with Syto 9 L7012 Component A, a DNA stain (Invitrogen Corp., Carlsbad, CA). A confocal laser scanning microscope (Leica TCS SP5 microscope with a HCX PL APO 100x (NA 1.4) objective) was used to examine the stained biofilms. The generated CLSM images were processed using Leica LAS AF software (Leica Microsystems GmbH, Wetzlar, Germany) to produce three-dimensional and cross-section representations of the biofilms. Statistical analyses of the biofilms (thickness, coverage and roughness) were conducted using the PHLIP software (Mueller et al., 2006).

### **Attachment assays**

Attachment assays on graphite, glass and Fe(III) oxide-coated glass were conducted as described in Chapter 2. Briefly, soluble electron donor and acceptor (10 mM acetate and 40 mM fumarate) were provided for cells exposed to glass or graphite surfaces for the entire course of the experiment. However, cells grown on Fe(III) oxide-coated glass had access to soluble electron donor and acceptor for one day, and to only soluble electron donor for the following four days to force the cells to use the Fe(III) oxide particles coating the glass surface as the electron acceptor. Fe(III) oxide-coated borosilicate cover slips were prepared as described elsewhere (van Schie and Fletcher, 1999).

## **Results**

### **Qualitative transcription analyses of the *pilA* gene in the short (*pilA4*(M1)) and long (*pilA5*(RBS)) isoform strains**

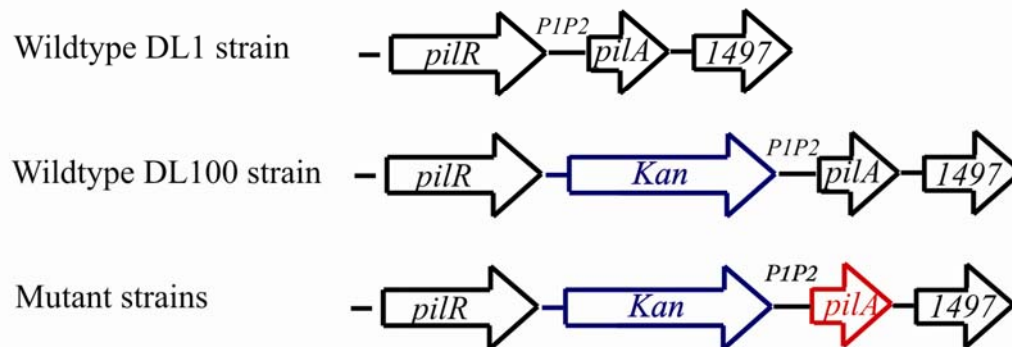
To test the hypothesis that two PilA isoforms exist and to determine the function of each preprotein variant, two mutant strains were generated. The *pilA4*(M1) strain had point mutations that changed the predicted translation start codon TTG of the long isoform into a nonstart codon CCC. These mutations were upstream of the start site for the short isoform transcript of *pilA*, and therefore, were not expected to affect the amino acid sequence of the short isoform PilA preprotein (Figure 16). In the long isoform *pilA5*(RBS) strain, mutations were introduced in the ribosomal binding site of the short *pilA* transcript. This strategy was favored over introducing point mutations in the internal translation start codon, because the corresponding amino acid would be altered in the long PilA isoform (Figure 16). Instead, base pairs were changed in a way that disrupted the ribosome-binding site, but maintained a long isoform sequence identical to the wildtype (Figure 17A) using codons that occur with similar frequency in the *G. sulfurreducens* genome. Both mutant strains were selected by means of a kanamycin resistance cassette located before the P1 and P2 promoters of the *pilA* gene (Figure 17B). The isogenic control strain DL100, having a kanamycin resistance marker in the same location relative to wildtype *pilA*, was isolated and confirmed to have no defect in expression of PilA (*data not shown*).

To determine whether the introduced mutations had any effect on transcription of the *pilA* gene, qualitative reverse transcriptase PCR reactions were performed on DNA-free RNA samples extracted from strains *pilA4*(M1) and *pilA5*(RBS) and the wildtype. PCR reactions were conducted using the synthesized cDNA as a template and a set of

primers designed to amplify the long *pilA* transcript; the forward primer consisted of a sequence upstream of the transcription initiation site of the short *pilA* transcript (Figure 18A). A positive signal for the long transcript was detected in all mutant strains at levels comparable to the wildtype strain (Figure 18B).

**A**

Mutant strain	Native sequence	Mutations
<i>pilA4</i> (M1)	TTG M	CCC P
<i>pilA5</i> (RBS)	AGA AGA <i>AAG</i> GAG ACA R R K E T	CGT CGT AAA GAA ACT R R K E T
<i>pilA6</i> (G20L)	AAA GGT K G	AAG CTT K L

**B**

**Figure 17.** Illustration of the *G. sulfurreducens* mutant strains used in Chapter 3. (A) Depiction of the DNA sequence change in each mutant strain. The ribosome binding site is italicized. The amino acids corresponding to the native and the mutant codons are indicated. TTG usually codes for leucine but in this case it is a translation start codon and is translated as methionine. (B) The kanamycin resistance cassette was introduced 5' to the P1 and P2 promoters region. The *pilA* gene is wildtype in strains DL1 and DL100 (depicted in black) and carries point mutations in the *pilA4*(M1), *pilA5*(RBS) and *pilA6*(G20L) mutant strains (depicted in red).

A

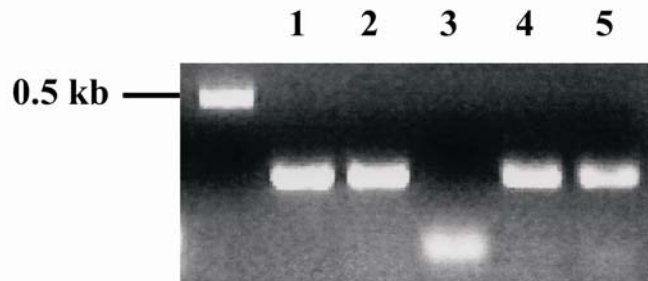
```

TTGGCCAATTACCCCATACCCCAACACAAGCAGCAAAAAGAAGA
AAGGAGACACTTATGCTTCAGAACTCAGAAACAGGAAAGGTTTC
ACCCCTTATCGAGCTGCTGATCGTCGTTGCGATCATCGGTATTCTC
GCTGCAATTGCGATTCCGCAGTTCTCGGCGTATCGTGTCAAGGCG
TACAACAGCGCGGCGTCAAGCGACTTGAGAAACCTGAAGACTGCT
CTTGAGTCCGCATTTGCTGATGATCAAACCTATCCGCCCGAAAGT
TAA

```

↗ +1

B



**Figure 18.** Qualitative reverse transcriptase PCR reactions of the wildtype and the mutant strains. (A) DNA sequence of the entire *pilA* gene. The two potential start codons are colored in red. The transcription initiation site of the short transcript is indicated by an arrow. The ribosome-binding site of the short isoform is italicized and underlined. The forward and reverse primers used for the RT-PCR reactions are highlighted. (B) DNA gel of the RT-PCR reaction products. (1) Control using primers specific for the *proC* gene with cDNA from wildtype strain. RT-PCR with primers specific for *pilA* with cDNA from the (2) wildtype, (3)  $\Delta(pilA)2$ , (4) *pilA4*(M1) and (5) *pilA5*(RBS) strains.

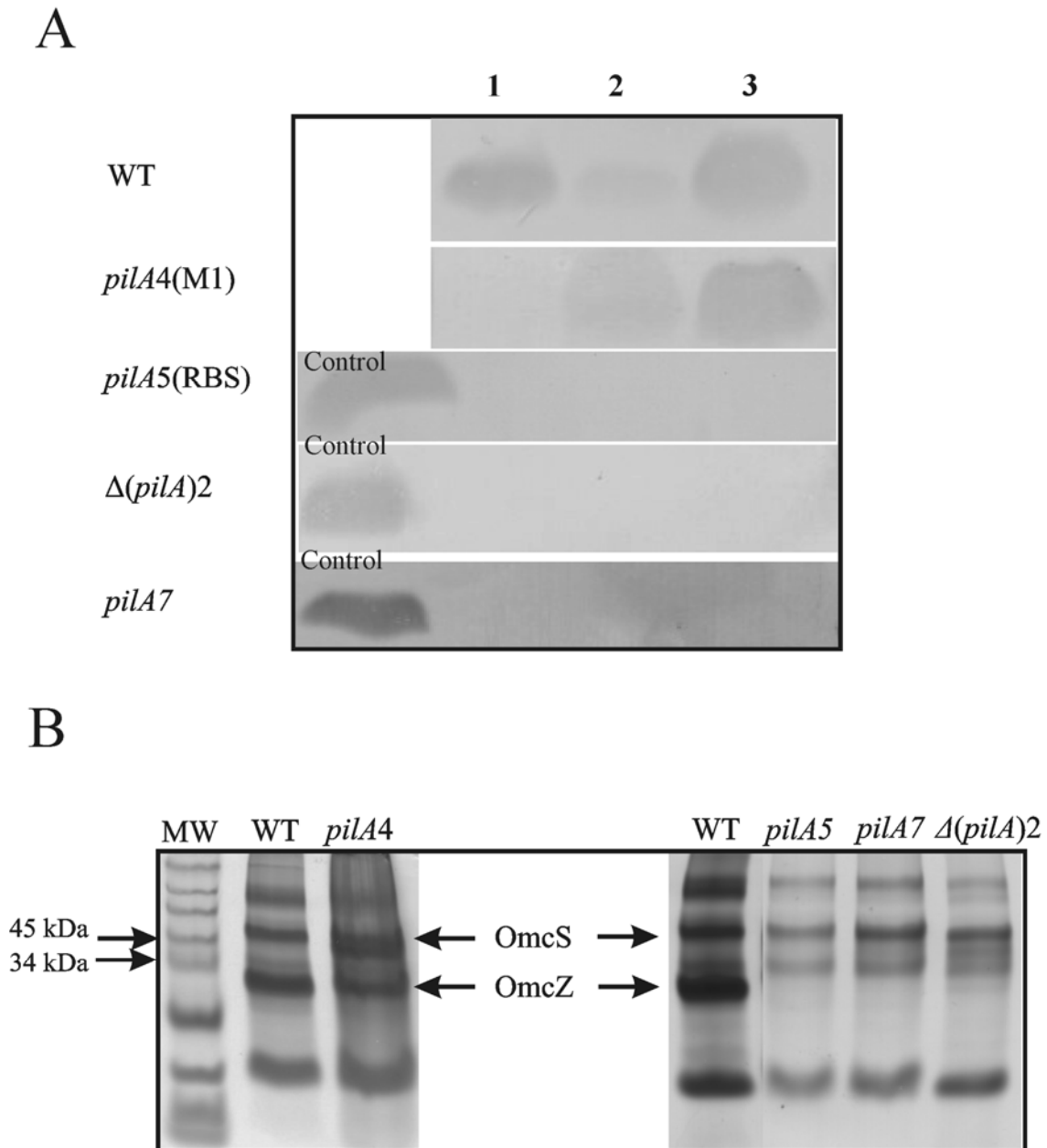
**Analysis of the short-isoform-only (*pilA4* (M1)) and the long-isoform-only (*pilA5* (RBS)) strains provides evidence of two PilA preprotein isoforms and cooperation among isoforms**

To evaluate the effect of each of the introduced mutations on the expression of *pilA*, the presence and the location of the PilA protein were determined by Western blot. In the wildtype strain DL1, as well as in the isogenic control with wildtype *pilA* strain, DL100, PilA is usually detected in three distinct fractions. The protein is found to be secreted (presumed to be assembled into filaments) and also located inside the cell as a soluble and a membrane-associated (sedimentable) protein. In the short isoform (*pilA4*(M1)) strain, where the long isoform's translation start codon is mutated, no PilA protein was detected by Western blot in the supernatant fraction, but PilA was still present in the cell-associated fractions 1 and 2 (Figure 19A). This result suggests that the TTG start codon is indeed functional and the long isoform is necessary for secretion of PilA and its assembly into the filamentous structure. The similar PilA band intensities of the *pilA4*(M1) strain and wildtype suggest that the amount of PilA protein inside the cell can be attributed entirely to a short PilA isoform, with ATG as the start codon. If the two preprotein isoforms functioned independently, one would expect to see only secreted PilA protein when the short isoform was eliminated. However, neither secreted nor intracellular protein was detected when the ribosome-binding site of the short isoform was mutated (Figure 19A). The absence of PilA protein derived from the long preprotein isoform could not be attributed to a transcriptional defect (compare Figure 18B), nor to a change in protein structure because our strategy maintained the amino acid sequence of the protein as it is in the wildtype (Figure 17A), using codons of similar frequency. This result indicates that the PilA protein derived from the long isoform is neither secreted nor



accumulated intracellularly without the cooperation of the short isoform. The *pilA* null strain, *pilA7*, with mutations of both the long isoform's translation start codon and the short isoform's ribosome-binding site, exhibited, as predicted, no PilA protein in any of the three subcellular fractions (Figure 19A).

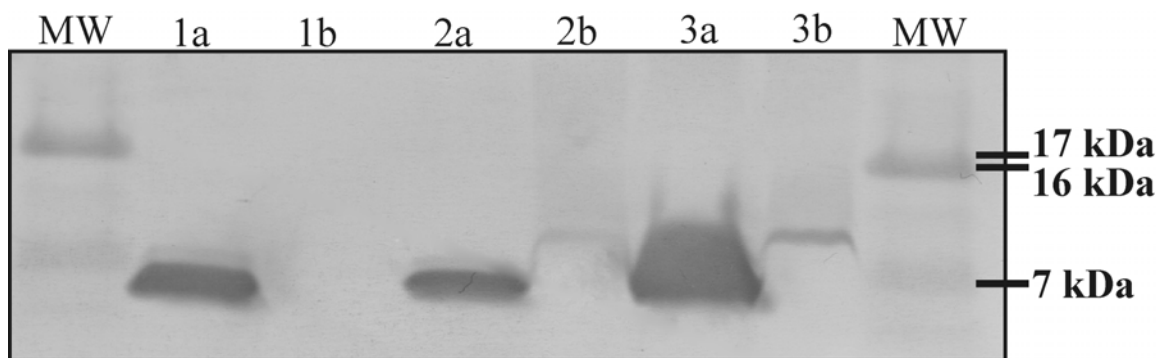
Besides the secretion of PilA, the secretion of other extracellular proteins also appears to be affected by the absence of the short PilA isoform. In heme-stained gels, OmcZ, which is important for extracellular electron transfer (Nevin et al., 2009; Richter, 2009), was not detected among the outer cell surface proteins of the long isoform (*pilA5*(RBS)) strain; this observation was also made for the *pilA* deletion strain ( $\Delta$ (*pilA*)2) and for the *pilA* null strain (*pilA7*), in which translation of both isoforms is disrupted (Figure 19B). On the other hand, proper localization of the outer surface cytochromes was observed for the short isoform (*pilA4*(M1)) strain and in levels comparable to the wildtype (Figure 19B). The short PilA isoform, being present in the *pilA4*(M1) strain, suggests that the mislocalization of OmcZ in the long isoform (*pilA5*(RBS)), the null (*pilA7*) and the PilA-deficient strains is attributable to the absence of the short PilA isoform.



**Figure 19.** Characterization of the *pilA4*(M1), *pilA5*(RBS) and *pilA7* mutant strains. (A) Western blot analysis of the wildtype (WT), *pilA4*(M1), *pilA5*(RBS),  $\Delta$ (*pilA*)2 and *pilA7* mutant strains using the PilA-specific antibody. PilA fractions: (1) supernatant fraction, (2) cell-associated fraction 1 and (3) cell-associated fraction 2. All PilA protein fractions migrated at 7 kDa. (B). Heme-stained SDS-PAGE of loosely bound outer surface *c*-type cytochromes prepared from the wildtype (WT), *pilA4*(M1), *pilA5*(RBS), *pilA7* and  $\Delta$ (*pilA*)2 strains. Each lane contains 3  $\mu$ g of total protein.

### **The long and short PilA preproteins are processed into a single mature form**

Type IV pilins are synthesized as prepilins with a leader sequence that is recognized and cleaved by a specific peptidase (PilD) following a conserved amino acid, glycine -1 (Strom and Lory, 1991). Despite the fact that the long PilA preprotein isoform has 20 more amino acids at the N-terminus, which corresponds to a 9 kDa molecular weight instead of the 7 kDa weight of the short preprotein isoform, no reduced migration was detected in any of the Western blots for any of the protein fractions (Figure 19A). This finding is attributed to the activity of peptidase PilD at the inner membrane, whereby both isoforms are cleaved at glycine -1 (Arts et al., 2007; Francetic et al., 2007), resulting in the same mature protein. To test this hypothesis a point mutation in the peptidase cleavage site (glycine -1) was introduced in the genomic copy of *pilA*, in which the final glycine of the prepeptide was replaced by leucine (Figure 17A). The genomic *pilA* sequence was verified to have no other mutations but the leucine mutation. The mutant strain, *pilA6*(G20L), was cultured under the same conditions as the wildtype and the protein samples were prepared and fractionated as described in the Materials and Methods to isolate subcellular fractions containing the secreted and cell-associated PilA protein. According to the Western blot analyses (Figure 20), no secreted PilA was detected for the mutant *pilA6*(G20L), suggesting the requirement for protein cleavage to get secreted. Interestingly, a shift in PilA migration corresponding to 9 kDa was detected in the intracellular fractions of the mutant cells, supporting our hypothesis that the preprotein of 9 kDa can only be detected when either the enzymatic activity of PilD is altered or the cleavage site in the PilA preprotein is disrupted.



**Figure 20.** Western blot analysis of the *pilA6*(G20L) mutant strain. PilA fractions are (1) supernatant fraction, (2) cell-associated fraction 1 and (3) cell-associated fraction 2 of the (a) wildtype and (b) *pilA6*(G20L) strains.

### **The short isoform strain displays diminished current production in microbial fuel cells**

Previously, it was reported that the PilA-deficient strain is severely impaired in current production in microbial fuel cells (Reguera et al., 2006). However, subsequent investigations of the distribution of the outer surface cytochromes within PilA-deficient cells<sup>\*</sup> revealed the unexpected mislocalization of one of the cytochromes important for current production, OmcZ (Nevin et al., 2009). These findings led to the question of whether the reduction in current production observed in the earlier study (Reguera et al. 2006) is due to an additive effect of the absence of type IV pili and OmcZ from the cell outer surface.

The short isoform (*pilA4*(M1)) strain lacks PilA protein in the secreted fraction; in other words, no filamentous PilA is present. However, this strain still has a proper distribution of the outer surface cytochromes (including OmcZ) in comparable levels to those in the wildtype (Figure 19B). Therefore, the current produced by biofilms of the short isoform (*pilA4*(M1)) strain was measured in a microbial fuel cell to assess the

<sup>\*</sup> Izallalen and Lovley- Unpublished observations

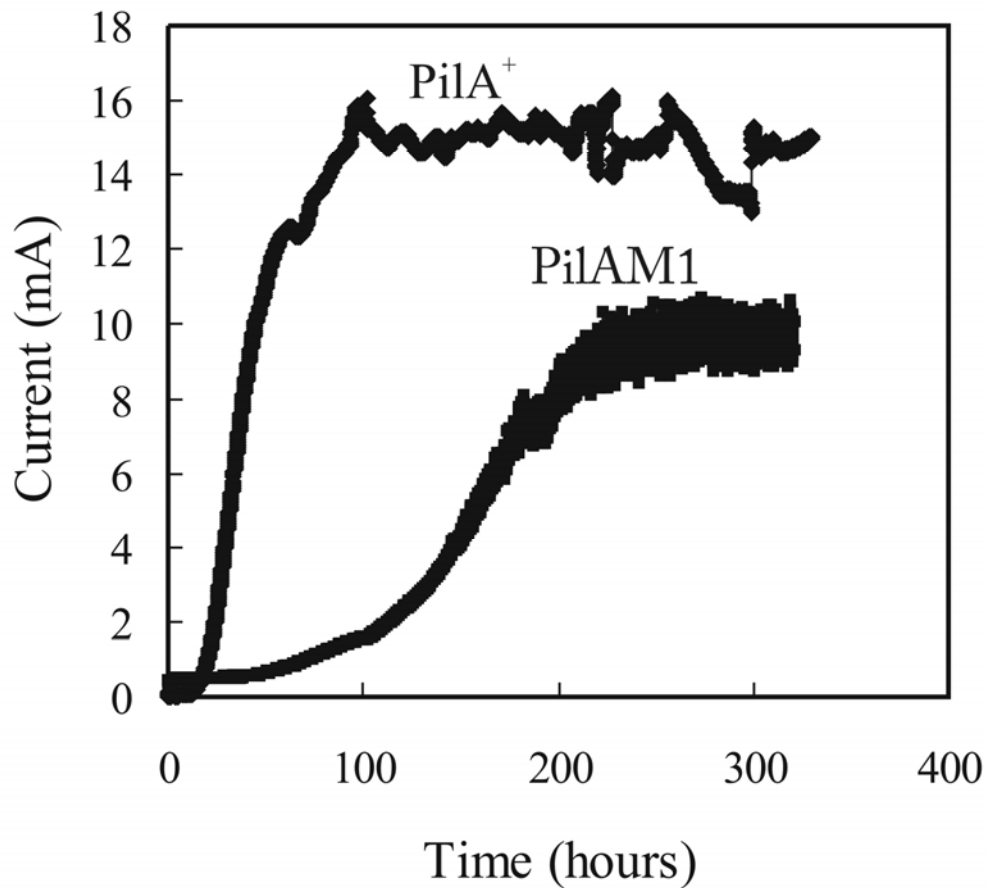
requirement of type IV pili alone for current production. The experimental conditions were set as described in the Materials and Methods section. The strains were fed with acetate, and a graphite electrode poised to +300 mV (vs. an Ag/AgCl reference electrode) served as the sole electron acceptor. The system was switched to a continuous supply of medium containing 10 mM acetate once current production was initiated. Current production by the short isoform (*pilA4*(M1)) strain followed a different pattern than that of the wildtype DL100 (Figure 21). In the wildtype strain DL100, current production sharply increased and reached a maximum current of  $15.02 \pm 0.49$  mA, which is typical of the wildtype strain (DL1) under the same experimental conditions (Nevin et al., 2009). Thus, the kanamycin cassette did not interfere with secreted PilA function. By comparison, the maximum current produced by the short isoform strain ( $10.4 \pm 0.6$  mA) was lower than DL100 (Figure 21) or DL1 (Nevin et al., 2009) and it took significantly longer to achieve the maximum current (~200 h) in comparison to either DL100 (~100 h, Figure 21) or DL1 (Nevin et al., 2009).

Confocal microscopy was used to assess the structures of biofilms formed by wildtype and mutant, which could account for the differences in current production. After three days at maximum current, confocal microscope images were taken of biofilms from five random locations in each of three independent experimental samples for each strain (15 images per strain). Biofilm thickness and substrate coverage of the short isoform and the isogenic control strains were determined. The results are summarized in Table 6. The anode biofilm formed with the short isoform strain had a greater maximum thickness (72  $\mu$ m) compared to a maximum thickness (55  $\mu$ m) of the isogenic wildtype DL100 biofilm, but the short isoform *pilA4*(M1) biofilm had less surface coverage compared to

wildtype (38% vs. 67%). Considered together, the average biofilm thicknesses were comparable, but the short isoform *pilA4*(M1) biofilm had a greater roughness compared to wildtype. These differences are reflected in the representative confocal images of Figure 22. The projection images show that the short isoform biofilm covers a smaller fraction of the anode surface and the cross-section images show that the short isoform biofilm contains taller pillar structures with deeper channels between the pillars. The differences in biofilm morphology, especially the smaller percent coverage displayed by the short isoform *pilA4*(M1) strain provides an explanation for the difference in current.

Type IV pili of *G. sulfurreducens* have been previously shown to be electrically conductive (Reguera et al., 2005). Our observation, that the maximum current at the anode is reduced when these pili are absent (Figure 21) is consistent with this requirement (Reguera et al., 2006; Nevin et al., 2009; Richter, 2009). But a significant difference between the previous studies and the present work resides in the fact that the previous studies were conducted with mutants that lack Type IV pili *and* have an aberrant distribution of outer membrane cytochromes (Figure 19), while the short isoform strain lacks Type IV pili yet has a wildtype-like Omc distribution, so that OmcZ – a critical component of electron transfer within the biofilm bulk (Nevin et al., 2009; Richter, 2009), is still present (Figure 19). Thus, the current production by the short isoform strain – lower than wildtype, but greater than the PilA-deficient strains studied previously (Reguera et al., 2006; Nevin et al., 2009), is plausible and can be explained by the absence of type IV pili. From investigations conducted with other Gram-negative species, it is known that type IV pili mediate cell attachment to surfaces (O'Toole and Kolter, 1998; Ward and Zusman, 1999). The differences between the biofilms (Figure 22)

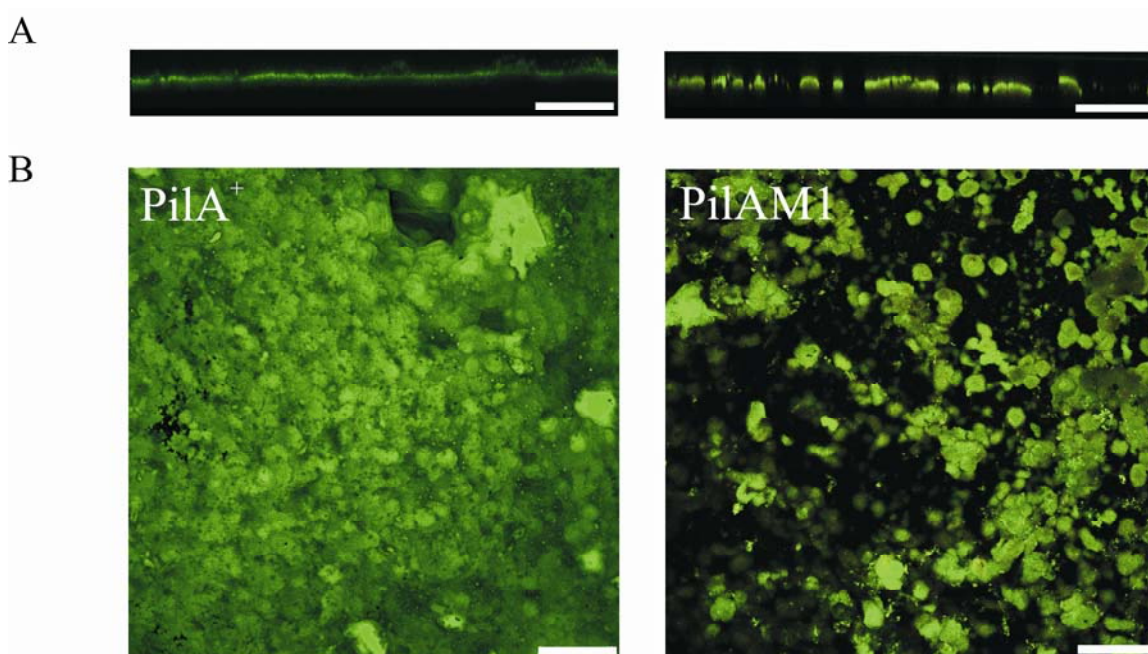
generated with the wildtype and short isoform strains are consistent with these observations, and we also suspect that the longer delay in current production in the absence of secreted PilA is a consequence of compromised cell attachment (see next section). However, as biofilms *do* form with the short isoform strain, it seems that secreted PilA, while important, is not an absolute requirement for cell attachment, biofilm growth and current production.



**Figure 21.** Current production by the wildtype DL100 (PilA<sup>+</sup>) and the *pilA4*(M1) mutant (PilAM1) strains in microbial fuel cells. Cells were grown on a potentiostat-poised graphite electrode with strictly anaerobic media with acetate as electron donor. Electrodes were poised at +300 mV (vs. an Ag/AgCl reference electrode). Data are representative of three biological replicates.

Strain	Substratum coverage (%)	Thickness	Roughness	Maximum thickness ( $\mu\text{m}$ )
Wildtype DL100	$67 \pm 7$	$44 \pm 4$	$0.26 \pm 0.07$	55.00
<i>pilA4</i> (M1)	$38 \pm 12$	$47 \pm 13$	$0.49 \pm 0.08$	72.00

**Table 6.** Quantitative analysis of the mature biofilms of the wildtype DL100 and the short isoform *pilA4*(M1) strains grown on poised graphite electrodes in microbial fuel cells and producing maximum current for three days. Values are the average  $\pm$  the standard deviation of triplicates.



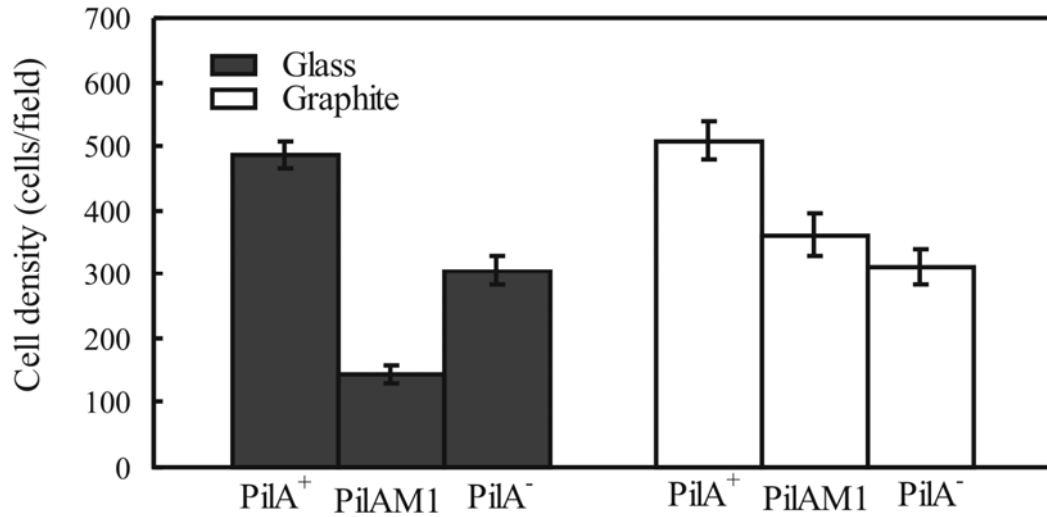
**Figure 22.** Confocal scanning micrographs of the isogenic wildtype DL100 (PilA<sup>+</sup>) and the *pilA4*(M1) (PilAM1) biofilms formed on graphite anodes and producing maximum current for 3 days in microbial fuel cells. (A) Cross-sectional and (B) top-down three-dimensional views of the biofilm. Cells were treated with fluorescent nucleic acid-binding stain before images were obtained. Scale bars, 250  $\mu\text{m}$ .



## Evaluation of the structural role of secreted PilA in biofilm formation

Type IV pili are known to be an important structural component for surface adhesion and colony formation (Shi et al., 1996; O'Toole and Kolter, 1998; Ward and Zusman, 1999). In *G. sulfurreducens* type IV pili have been demonstrated to be essential for attachment to glass surfaces (Reguera et al., 2007). We examined the short isoform *pilA4*(M1) strain for the ability to attach to graphite and glass surfaces, because this strain is different from the PilA-deficient strain used in previous investigations (Reguera et al., 2007): the outer surface of the short isoform strain created for this study only lacks secreted PilA, but seems to maintain the wild-type-like cytochrome composition. The short isoform *pilA4*(M1) strain and the wildtype DL100 were incubated in strictly anaerobic freshwater medium with acetate as the electron donor and fumarate as the electron acceptor, at the pilus production-inducing temperature, 25°C, and in the presence of graphite or glass slips for four days. CLSM image stacks were collected from a minimum of five random locations for each of three biological replicates within each experiment. The absence of secreted PilA had a negative impact on cell attachment to surfaces regardless of the surface material. The short isoform *pilA4*(M1) cells exhibited a significantly lower attachment to graphite, by an average of 160 fewer cells per field relative to wildtype (DL100) cells (unpaired t-test  $p < 0.01$ ), which exhibited on average  $490 \pm 180$  cells/field (Figure 23). Also, the attachment of the short isoform strain to glass was substantially lower, by 340 cells/field, compared to DL100 (unpaired t-test  $p < 0.01$ ), which exhibited on average  $470 \pm 130$  cell/field (Figure 23). These data are consistent with the published observations made with PilA- and PilR-deficient strains (Reguera et al., 2007; Juarez et al., 2009). Taken together, these data provide further support of an

active role for pili in surface colonization, even when the surface is not an electron acceptor.

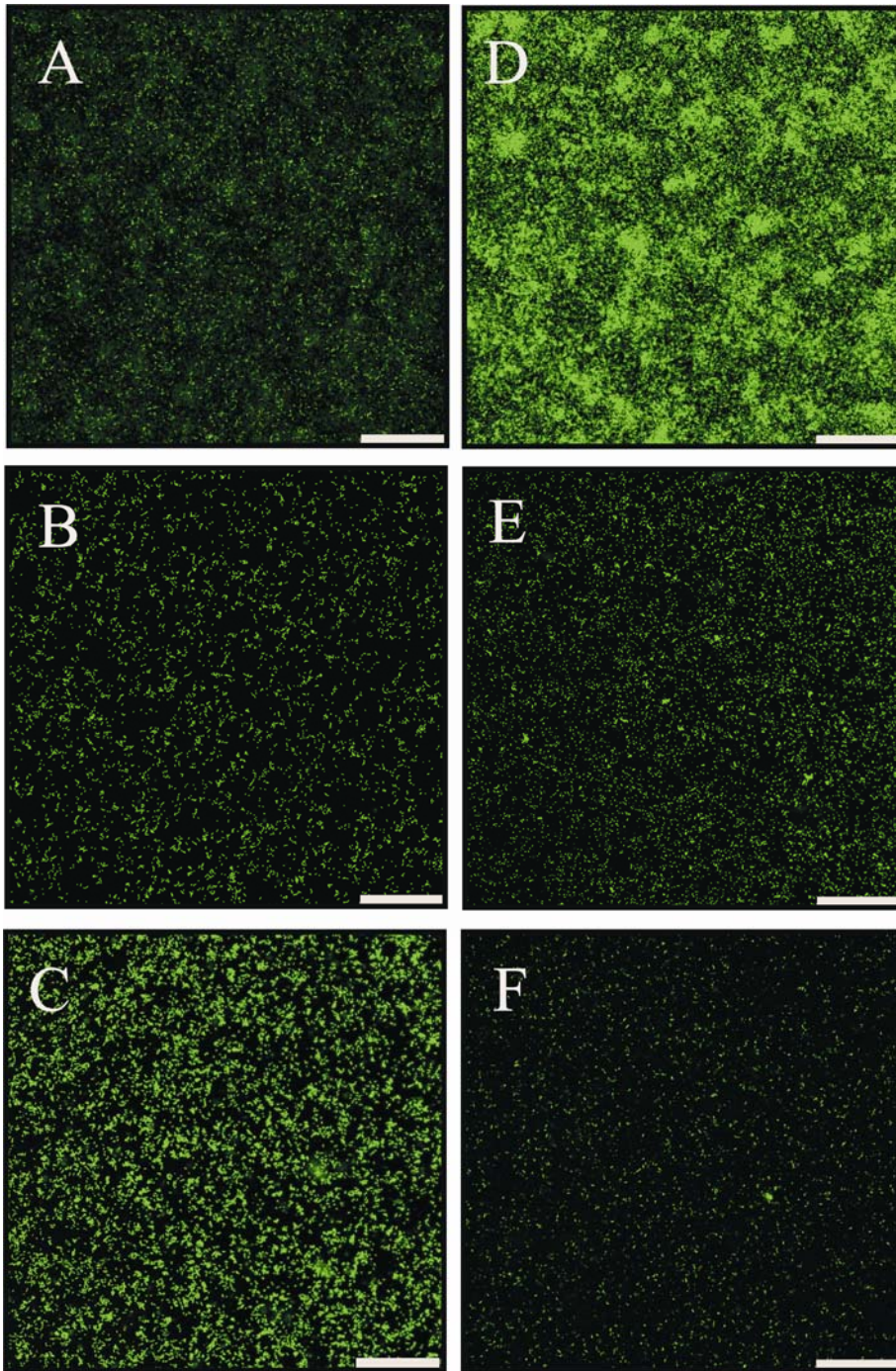


**Figure 23.** Average biomass of the mature wildtype DL100 (*PilA*<sup>+</sup>), the *pilA4*(M1) (*PilA*M1) and the *Δ(pilA)2* (*PilA*<sup>-</sup>) biofilms formed on glass (solid bars) and graphite (open bars) surfaces. Cells were grown in freshwater fumarate-acetate media at 25°C under strictly anaerobic conditions. The results are the average of six biological replicates from two independent experiments. Error bars are standard errors of mean.

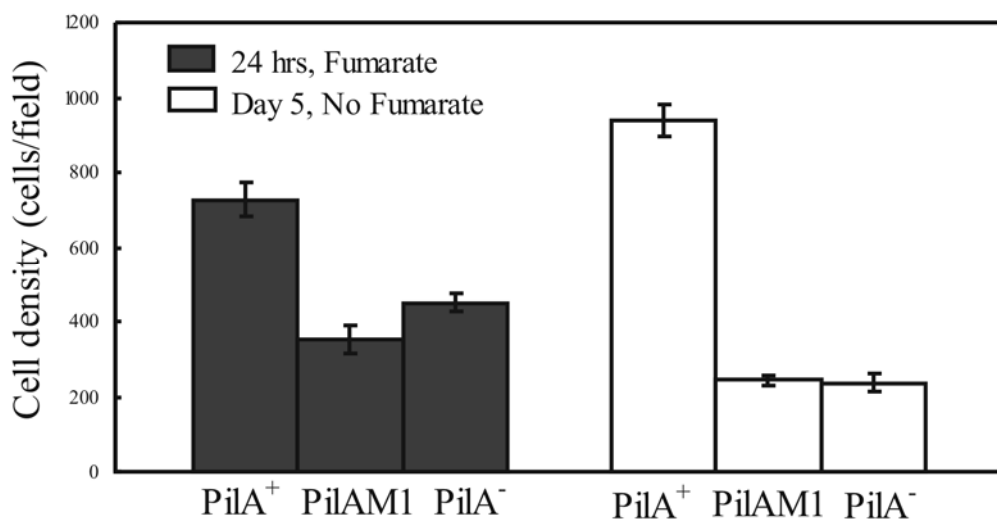
### Secreted *PilA* is important for growth on insoluble Fe(III) oxide

The *pilA4*(M1) strain was examined for the ability to grow on an insoluble electron acceptor. Cell growth was initiated on Fe(III) oxide-coated glass slides in the presence of a soluble electron donor and a soluble electron acceptor. After an initial 24-hour growth period, the soluble electron acceptor was removed, so that insoluble Fe(III) oxide was the only acceptor. The representative CLSM images in Figure 24 and the average cell densities presented in Figure 25 of the wildtype DL100, the short isoform *pilA4*(M1) and the *pilA* deletion *Δ(pilA)2* strains illustrate the similarities and differences in the attachment and growth of these strains on Fe(III) oxide-coated glass. Figure 24 shows that wildtype and mutant cells were capable of attachment to the Fe(III) oxide-

coated glass when both soluble electron donor and acceptor were provided (Figure 24A-C). However, attachment was less by an average of 370 cells/field (unpaired t-test,  $p < 0.01$ ) compared to DL100, which exhibited an average of  $690 \pm 200$  cells/field (Figure 25, gray bars). This observation was consistent with earlier reports of a PilA-deficient strain that, while still able to access Fe(III) oxide particles (Afkar et al., 2005), generated a biofilm on Fe(III) oxide-coated glass in the presence of soluble electron acceptor with a thickness ca. 50% smaller than the biofilms of wildtype cells (Reguera et al., 2007). Significant growth was noted with the isogenic wildtype cells after removing fumarate from the medium, and incubating the cells with Fe(III) oxide as the sole electron acceptor (compare Figure 24A and D). This was quantified as an average increase of 230 cells per field (unpaired t-test  $p < 0.01$ , Figure 25). By contrast, the short isoform strain was not able to use Fe(III) oxide particles as an electron acceptor. No growth was detected in any of the six biological replicates; instead, the average number of cells/field decreased by 80 (unpaired t-test  $p < 0.05$ , Figure 24B, 24E and 25). The deletion strain ( $\Delta pilA$ ) was similarly defective in growth in the absence of a soluble electron acceptor (Figure 24C, 24F and 25). These data are consistent with the previous report for the PilA-deficient strain (Reguera et al., 2007); the short isoform strain, which does not secrete PilA, and the PilA-deficient strain are both incapable of using Fe(III) oxide-coated as an electron acceptor.



**Figure 24.** Confocal microscopic analyses of the wildtype DL100 (A and D), the *pilA4*(M1) (B and E) and the  $\Delta(pilA)2$  (C and F) biofilms formed on Fe(III) oxide-coated glass. Images were taken after incubation in the presence of 40 mM fumarate for 24 hours (A, B and C) and after removal of the fumarate and incubating the cells without a soluble electron acceptor for 4 days (D, E and F). Scale bars, 75  $\mu$ m.



**Figure 25.** Average cell growth of the wildtype (PilA<sup>+</sup>), the *pilA4*(M1) (PilAM1) and the  $\Delta$ (*pilA*)2 (PilA<sup>-</sup>) biofilms formed on Fe(III) oxide-coated glass surfaces. Cell densities were measured after 24 hours of incubation in the presence of fumarate (solid bars) and after 4 days of incubation with no soluble electron acceptor (open bars). The results are the average of six biological replicates from two independent experiments. Error bars represent standard error of mean.

## Discussion

Type IV pili of *Geobacter sulfurreducens* are an essential component of the conductive anode biofilm, promoting cell-to-cell electron transfer through the 50  $\mu$ m thick biomass layer (Franks et al., 2009; Nevin et al., 2009; Richter, 2009). The biofilms are electrically conductive and there is evidence that type IV pili are the final electron conduit onto insoluble Fe(III) oxide (Afkar et al., 2005). The *pilA* gene, encoding the pilus structural subunit protein PilA, is 273 bp long in total (starting from the TTG translation start codon). Transcription of *pilA* is regulated by a two-component regulatory system composed of the histidine kinase, PilS, and the response regulator, an RpoN-dependent enhancer-binding protein, PilR (Juarez et al., 2009). Previous analysis of the

transcription initiation site of *pilA* revealed the presence of two transcripts of the same gene, a long transcript that is RpoN-dependent and a short alternative non-RpoN-dependent transcript (Juarez et al., 2009). Each transcript had an independent transcription start site, a potential ribosomal binding site and a predicted translation start codon, which suggested the presence of two PilA preprotein isoforms encoded by the same *pilA* gene.

We explored the hypothesis of two PilA isoforms, and investigated the roles that these two putative isoforms may have, by generating mutant strains to eliminate one isoform at a time. The expression and localization of PilA was detected using Western blots probed with an anti-PilA antiserum raised against a peptide of the C-terminus of the PilA protein. The anti-PilA antibody was shown to be protein specific and was used to detect the expression and over production of PilA protein (hyperpiliation) in *Geobacter sulfurreducens* strains (Nevin et al., 2009; Yi et al., 2009; Klimes et al., 2010). Electron microscopy approach could not be used as a method of detection due to the unavailability of an antibody specific to the nanowires consisting of multiple PilA-subunits, and to the finding that *G. sulfurreducens* possesses several other filament-like structures that have the same morphology in length and diameter as type IV pili (Klimes et al., 2010).

When the translation start codon of the long isoform was mutated (TTG into CCC), no secreted PilA protein was detected by Western blot. However, PilA protein was present inside the cell in soluble and membrane fractions. These data indicate that the TTG start codon is functional and produces a preprotein isoform that is essential for PilA secretion and/or export to the outer surface of the cell. Heme-stained gels of the outer membrane cytochromes of the short isoform *pilA4*(M1) strain revealed a wildtype-like

composition of the outer membrane *c*-type cytochromes (OmcS and OmcZ) necessary for Fe(III) oxide reduction and generation of electricity in microbial fuel cells (Mehta et al., 2005; Nevin et al., 2009; Richter, 2009). On the other hand, disruption of expression of the short isoform (in the long-isoform-only *pilA5* strain) eliminated both isoforms according to Western blot analysis. Despite the fact that the long isoform was still transcribed, no PilA protein was detected in any of the cell fractions: supernatant fraction, cell-associated fraction 1 or cell-associated fraction 2, indicating that expression of the short isoform is necessary for the stability of the long isoform. The *c*-type cytochrome OmcZ was not localized on the outer surface in the long isoform *pilA5*(RBS) strain, a phenotype that was also observed for the PilA-deficient strain; suggesting that this phenotype is attributable to the absence of the short PilA isoform with the start codon ATG.

The PilA-deficient strain lacks proper localization of some *c*-type cytochromes on the outer cell surface\*, cytochromes that are important for extracellular electron transfer (Nevin et al., 2009; Richter, 2009). These observations prompted us to examine a short-isoform-only (*pilA4*) strain that did not secrete the mature PilA required for filament production. Despite the fact that the short isoform *pilA4*(M1) strain lacked extracellular PilA, the outer membrane cytochromes were distributed properly, which was not the case for  $\Delta pilA$  strains. We were therefore able to study the role of pili in attachment, biofilm formation and extracellular electron transfer to insoluble electron acceptors, without the additional influences that mislocalized cytochromes may contribute. The absence of extracellular PilA affected the cells' ability to attach to surfaces: while the short isoform

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\* Izallalen and Lovley- Unpublished observations.

*pilA4*(M1) cells exhibited a moderate decrease in attachment to graphite surfaces with soluble electron donor and acceptor in the medium, attachment to glass was severely diminished under the same conditions. These data are consistent with previous findings that the PilA-deficient strain (in which both isoforms are deleted) and the PilR-deficient strain (in which the long *pilA* transcript is eliminated) were deficient in attachment to glass surfaces (Reguera et al., 2007; Juarez et al., 2009). Our data further identify the long PilA isoform initiated by the TTG translation start codon as the PilA isoform responsible for the attachment phenotype.

In microbial fuel cells, the short isoform *pilA4*(M1) strain was able to form a conductive biofilm on the graphite anode. However, the *pilA4*(M1) biofilm exhibited decreased anode surface coverage in comparison to the wildtype. The *pilA4*(M1) biofilm formed clusters on the graphite electrode surface with taller pillars, relative to the wildtype DL100 biofilm, and deeper channels between pillars. Due to the altered structure of the biofilm, mutant cells were more limited in accessing the graphite anode. The maximum current produced by the *pilA4*(M1) mature biofilm was  $10.4 \pm 0.6$  mA (vs.  $15.0 \pm 0.5$  mA for the wildtype DL100 biofilm). This could be attributed to missing type IV pili. Type IV pili of *G. sulfurreducens* were reported previously to serve to ‘wire’ cells together in the thick wildtype biofilm (Franks et al., 2009; Nevin et al., 2009; Richter, 2009). However, the observed current for the *pilA4*(M1) was higher than what was reported for the PilA-deficient strain (Reguera et al., 2006; Nevin et al., 2009), which could be explained by the proper localization of the outer surface cytochrome, OmcZ, in the short isoform *pilA4*(M1) strain. Deletion of the *omcZ* gene was previously reported to reduce severely the current production of cells grown in microbial fuel cells although



those cells possessed pili according to transmission electron microscopic analysis (Nevin et al., 2009). Confocal scanning microscopic images of the  $\Delta omcZ$  current-producing biofilm revealed a reduction in biofilm thickness with changes in its morphology (no pillar structures were observed) (Nevin et al., 2009). As OmcZ and PilA both contribute to biofilm structure and current production in biofilms of wildtype cells, it seems plausible that cells defective only in the production of pili, i.e. the short isoform strain, will generate more biofilm current than cells that are defective in the production of pili *and* in the distribution of OmcZ (a  $\Delta pilA$  strain).

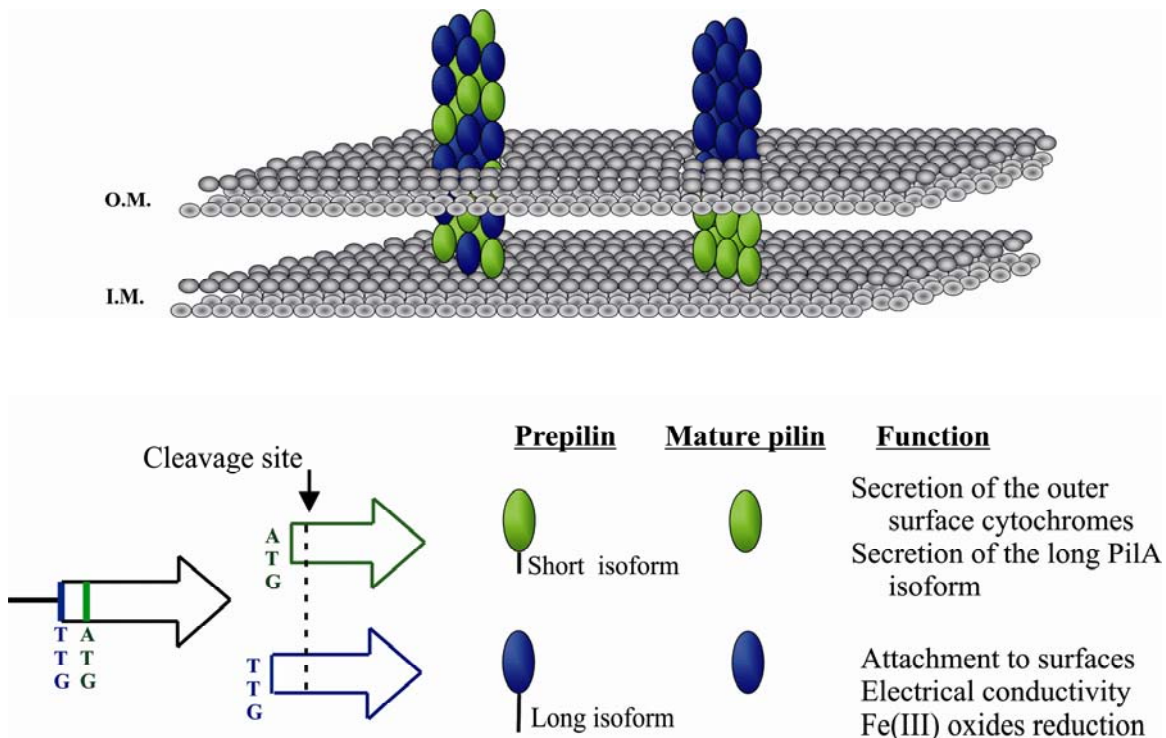
Regardless of whether both *pilA* transcripts were disrupted (in the PilA-deficient strain) or only the long transcript was absent (in the PilR-deficient strain), both mutant strains were incapable of growth with insoluble Fe(III) oxide in previous reports (Afkar et al., 2005; Reguera et al., 2007; Juarez et al., 2009). In our study, the short isoform *pilA4*(M1) cells were capable of attaching to Fe(III)-coated glass surfaces (to a somewhat lower extent compared to the wildtype), but they exhibited no growth when Fe(III) oxide was the sole electron acceptor. These data are consistent with the previously published data for the PilA-deficient and the PilR-deficient strains, confirming the importance of type IV pili in extracellular electron transfer independent of their role in the localization of *c*-type cytochromes.

## **Summary and conclusions**

Our data support the presence of two functional translation start codons of the *pilA* gene that lead to two preprotein isoforms, the long isoform and the short isoform (Figure 26). Both isoforms are cleaved at the same site (glycine position -1) leading to the same mature protein sequence. The mature PilA protein has the hydrophobic N-terminus known to form the core of the pilus fiber in homologous type IV pilins, In addition, the mature protein has a conserved phenylalanine and a glutamate at positions +1 and +5, respectively, which are believed to engage in a favorable electrostatic interaction between pilin subunits that stabilizes fiber assembly. The short isoform is required for secretion and stability of PilA and for secretion of the outer surface *c*-type cytochromes. The long isoform seems required only for the secretion of PilA. Mature PilA is presumably assembled into filamentous structures due to presence of all key elements required for pilus formation. The long preprotein isoform is required for cell attachment to various surfaces, for optimal current production and for extracellular electron transfer to insoluble Fe(III) oxide, processes in which pilus filaments may participate directly.

An operational model that is consistent with our observations is shown in Figure 26. Our data demonstrate that the short isoform is not secreted in the absence of the long isoform, suggesting that it may be directed exclusively to intracellular and membrane associated locations, such as the base of the pilus at the cell surface (Figure 26, right). This scenario implies that the long isoform is the only preprotein targeted for secretion and/or pilus formation. On the other hand, we must also consider the possibility that the pili are comprised of mature PilA protein derived from both the short and long preprotein isoforms (Figure 26, left). The long preprotein isoform is still a

requirement for pilus formation (and secretion), so that in its absence no secreted PilA is observed. However, the pilus formation permits the incorporation of both long and the short isoforms. Additional studies that can discriminate between the fates of the short and long are warranted.



**Figure 26.** Models proposed for assembly and function of the short and long PilA preprotein isoforms. The short and long isoforms are depicted in green and blue, respectively. Both isoforms are shown cleaved after glycine -1 once they reach the inner membrane, generating identical mature proteins. Model I (right) suggests that the mature PilA derived from the short PilA isoform remains intracellular and forms the base of the pilus fiber anchoring the long-isoform-derived PilA in the cell membrane. Model II (left) suggests that secreted PilA is derived from both preprotein isoforms, and long- and short-isoform-derived PilA are assembled into the intra- and extracellular regions of the pilus fiber.

## CHAPTER 4

### INVESTIGATION OF THE GENES REQUIRED IN *CIS* FOR EXPRESSION AND SECRETION OF GEOPILIN

#### Introduction

The nanowire hypothesis of *Geobacter sulfurreducens* has been of great interest to scientists due to its significance for bioremediation and generation of electricity (Reguera et al., 2005; Reguera et al., 2006; Reguera et al., 2007; Nevin et al., 2008; Franks et al., 2009; Richter, 2009). The conducting probe atomic force microscopy technique has been used to measure the conductivity across nanowires (putatively type IV pili of *G. sulfurreducens*) (Reguera et al., 2005). The results of this analysis suggested that structural associations of the pili with any of the redox-active outer surface cytochromes were absent under the applied growth and preparation conditions. The type IV pili appeared to be long, thin filaments and the PilA protein was determined to be the structural subunit of those pili (Reguera et al., 2005). Deletion of the *pilA* gene resulted in a strain,  $\Delta(pilA)1$ , that was incapable of growth on Fe(III) oxide although it was still able to attach to the Fe(III) oxide particles (Reguera et al., 2005; Reguera et al., 2007). Furthermore, the  $\Delta(pilA)1$  strain exhibited a deficiency in current production in microbial fuel cells (Reguera et al., 2006; Nevin et al., 2009).

The  $\Delta(pilA)1$  strain was later investigated for the pattern of its *c*-type cytochromes and their proper distribution on the cell's outer surface. The cytochromes OmcZ and OmcS appeared to be absent from the cells' surface and were determined to be localized intracellularly\*.

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\* Izallalen and Lovley- Unpublished observations.

Expression of the *pilA* gene in *trans* from the (pRG5\_*pilA*) plasmid restored Fe(III) oxide reduction partially (Reguera et al., 2005), attachment to glass surfaces completely (Reguera et al., 2007) and current production to the wildtype level (Nevin et al., 2009). However, subsequent investigations of PilA expression and secretion in the complemented strain revealed several unexpected findings:

- Semi quantitative RT-PCR revealed that, despite the fact that *pilA* expression from the pRG5\_*pilA* plasmid was regulated by the strong *tacUV5* & *lac* promoters, the *pilA* gene was only expressed at relatively low levels (Dr. A. Klimes, *personal communication*).
- DNA sequence analysis of the pRG5\_*pilA* plasmid indicated a clone of an N-terminally truncated sequence of the *pilA* gene. The *pilA* gene possesses two translation start codons; the external start codon TTG and the internal start codon ATG, which leads to two prepilin isoforms (see Chapter 3). The long PilA isoform, initiated by the TTG start codon, is essential for mature PilA to be secreted to the cell's outer surface and presumably to assemble into the filamentous pili. The short protein isoform is initiated by the internal start codon ATG and is detected only in the intracellular fraction in the absence of the long isoform. The pRG5\_*pilA* plasmid constructed by Reguera and co-workers (Reguera et al., 2005) harbors a sequence of the *pilA* gene starting from the internal start codon ATG. Our data in Chapter 3 confirmed that the external start codon TTG is a functional translation start codon and results in the long prepilin isoform. Therefore, expression of a truncated version of the gene with ATG as the

start codon (pRG5\_ *pilA*) will result only in the expression of the short prepilin isoform.

- The analysis of the composition of outer membrane *c*-type cytochromes in the genetically-complemented *pilA* mutant strain confirmed that OmcZ was secreted to the outer surface, but in higher levels than the wildtype, and that secretion of OmcS was not restored\*.

In this study, using various chromosomal *pilA* mutants, we investigated which features linked to the *pilA* gene are required for expression of PilA *in trans* to lead to a full restoration of expression and secretion.

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\* Izallalen and Lovley- Unpublished observations.

## Materials and methods

### Bacterial strains and plasmids

All *G. sulfurreducens* strains and plasmids used in this work are listed in Table 7.

Designation	Genotype or description	Source or reference
<b>Strains</b>		
DL1	Wildtype <i>G. sulfurreducens</i> strain	(Caccavo et al., 1994)
DL100	DL1, intact <i>pilA</i> ; Kan <sup>r</sup>	Chapter 2
$\Delta(pilA)2$	DL1, <i>pilA</i> in-frame deletion mutant; Kan <sup>r</sup>	Chapter 2
<i>pilA4</i> (M1)	DL1, <i>pilA</i> contains a TTG→CCC mutation of the long isoform's start codon; Kan <sup>r</sup>	Chapter 3
<i>pilA5</i> (RBS)	DL1, <i>pilA</i> contains synonymous mutations of the ribosome-binding site of the short isoform; Kan <sup>r</sup>	Chapter 3
DLRG9	DL1, the knockout mutant of GSU1497; Kan <sup>r</sup>	(Nevin et al., 2009)
<b>Plasmids</b>		
pLC1	A derivative of pRG5 (Kim et al., 2005) but without the <i>tacUV5</i> & <i>lac</i> promoters; Spec <sup>r</sup>	Chapter 2
pLC3	pLC1 containing the last 200 bp of GSU1495, the P1 and P2 promoters, and the entire <i>pilA</i> gene; Spec <sup>r</sup>	Chapter 2
pLC10	pLC1 containing the last 200 bp of GSU1495, the P1 and P2 promoters, <i>pilA</i> and GSU1497; Spec <sup>r</sup>	This work
pLC17	pLC1 containing genes <i>pilA</i> , GSU1497, and 1271 bp of GSU1498; Spec <sup>r</sup>	This work
pLC18	pLC1 containing genes <i>pilA</i> , GSU1497 to GSU1500; Spec <sup>r</sup>	This work
pLC19	pLC1 containing genes <i>pilA</i> , GSU1497 to GSU1505; Spec <sup>r</sup>	This work

**Table 7.** *Geobacter sulfurreducens* strains and plasmids used in Chapter 4

## **DNA manipulations**

Genomic and plasmid DNA purification and PCR reactions were performed as described in Chapters 2 and 3. Restriction enzymes and PCR primers were purchased as described in Chapters 2 and 3.

### **Construction of the *pilA* expression plasmid pLC10**

The last 200 base pairs of GSU1495 (the *pilR* gene), the P1 and P2 promoter region, the entire *pilA* gene and the downstream gene GSU1497 were amplified using genomic DNA of *G. sulfurreducens* as a template. The forward and reverse primers, rLC59F and rLC60R, were designed to introduce *Bam*H I and *Fsp* I restriction sites at the 5' and 3' ends of the amplicon, respectively. The amplified fragment and the pLC1 vector were double-digested at the *Bam*H I and *Fsp* I restriction sites, followed by ligation and transformation into *E. coli* chemical competent cells. The resulting plasmid was designated pLC10 and verified by DNA plasmid sequencing.

### **Construction of the *pilA* expression plasmid pLC19**

Plasmid pLC19 was constructed in three steps, each verified by PCR, digestion and sequencing. Primers were designed to amplify a 10.5 kb segment of *G. sulfurreducens* genomic DNA (containing all genes from GSU1496 to GSU1505) in three smaller fragments: fragment 1 (2.5 kb), fragment 2 (3.2 kb) and fragment 3 (5.2 kb) (Figure 27). The primers amplifying fragment 1 (rLC108 and rLC101) were designed to introduce *Fsp* I and *Xma* I restriction sites at the 5' and 3' ends respectively (Table 8), for ligation into vector pLC1 to create plasmid pLC17. The forward primer for amplification of fragment 2 (rLC102) was designed for a small overlap with fragment 1, including an *Nhe* I site unique within the 10 kb segment. The reverse primer (rLC103) was designed to

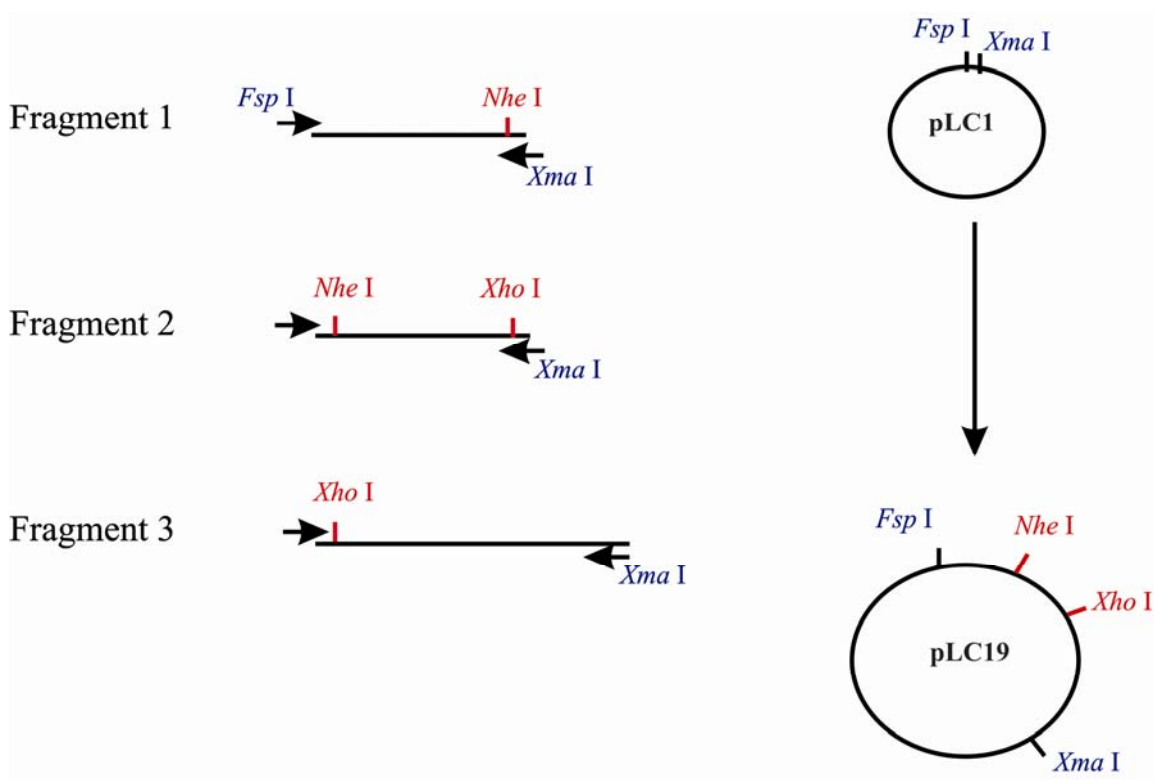


introduce an *Xma* I site at the 3' end of fragment 2 (Table 8). These two sites were used to insert fragment 2 into pLC17, creating plasmid pLC18. The forward primer for amplification of fragment 3 (rLC104) was designed for a small overlap with fragment 2, including an *Xho* I site unique within the 10 kb segment, and the reverse primer (rLC107) was designed to introduce an *Xma* I site at the 3' end of fragment 3 (Table 8). These two sites were used to insert fragment 3 into pLC18, creating plasmid pLC19. This plasmid contains the final 200 bp of GSU1495 (*pilR*), the P1 and P2 promoter regions, GSU1496 (*pilA*), GSU1497 and a cluster of genes on the 3' side of GSU1497 (GSU1498 to GSU1505). This plasmid was used to express the *pilA* gene in *trans* with various chromosomal *pilA* mutations.

Plasmids	Primers and sequence	Description
pLC10	<b>rLC59F:</b> 5'-GATAGGATCCGTCACCGAGTGCGAACTGCC-3'  <b>rLC60R:</b> 5'-CTATTGCGCAGAGGGCCATCTGGAA TCGGTAAC-3'	To amplify the last 200 bp of GSU1495 ( <i>pilR</i> ), the P1 and P2 promoter region, <i>pilA</i> and GSU1497. Restriction sites introduced ( <i>Bam</i> H I and <i>Fsp</i> I) are italicized and underlined.
pLC17	<b>rLC108:</b> 5'-GATATGCGCATCCACGCGGGTAAACGGCCTGA-3'  <b>rLC101</b> 5'-CTATCCCGGGTCTGCCGCATAACCGACGTAACTTC-3'	To amplify the last 200 bp of GSU1495 ( <i>pilR</i> ), the P1 and P2 promoter region, <i>pilA</i> , GSU1497, and the first 1271 bp of GSU1498 (fragment size is 2.5 kb). Restriction sites introduced ( <i>Fsp</i> I and <i>Xma</i> I) are italicized and underlined.
pLC18	<b>rLC102:</b> 5'-TGGTCATGTGTATAATCCCACCAAT TATGGC-3'  <b>rLC103</b> 5'-CTATCCCGGGGAGCCATTGGAGAGGACGACAG-3'	To amplify the last 1558 bp of GSU1498, GSU1499 and GSU1500 (fragment size is 3.2 kb). Restriction site introduced ( <i>Xma</i> I) is italicized and underlined.

pLC19	<b>rLC104</b> 5'-CTGTAGATCGTGACGGATTGGGT G-3'	To amplify GSU1501, GSU1502, GSU1503, GSU1504 and GSU1505 (fragment size is 5.2 kb). Restriction site introduced ( <i>Xma</i> I) is italicized and underlined.
	<b>rLC107</b> 5'-CTAT <u>CCCCGGG</u> ACATACCTGGCCACT GGTTC-3'	

**Table 8.** List of primers used in Chapter 4



**Figure 27.** Illustration of the strategy used for construction of the *pilA* expression plasmid pLC19. The plasmid was used for co-expression of the *pilA* gene, GSU1497 and the gene cluster at the 3' end in *trans* with chromosomal *pilA* mutant alleles.

## Attachment assays

Attachment assays on glass surfaces were conducted as described in Chapter 2. Cells were incubated on glass cover slips in freshwater medium with 40 mM fumarate and 10 mM acetate at the pilus expression-inducing temperature, 25°C. Biofilms were stained with Syto 9 L7012 Component A after four days of incubation and examined under a confocal laser scanning microscope as described in Chapter 2.

## Results

### Expression of the *pilA* gene from plasmid pLC3

To study the expression of the *pilA* gene in *trans* relative to its native chromosomal context, a new in-frame deletion mutant of *pilA* was first constructed as described in Chapter 2. The goal was to get an in-frame deletion version of the *pilA* gene with the kanamycin resistance cassette inserted before the P1 and P2 promoter region of *pilA* (Figure 28). This strategy was chosen to avoid any possible polar effect on the expression of genes at the 3' end, such as GSU1497, that might be regulated by the P1 and P2 promoters. The resulting strain in which the first sixteen and last ten codons of the *pilA* gene were fused in-frame, was designated  $\Delta(pilA)2$ . As expected, no PilA protein was detected by Western blot for the  $\Delta(pilA)2$  strain (Figure 29). The isogenic control with wildtype *pilA*, strain DL100 (Figure 28), was checked for any expression phenotype that might have resulted from insertion of the kanamycin resistance cassette before the P1 and P2 promoter region. Western blot analysis confirmed expression and secretion of PilA in the DL100 strain\* (Figure 29).

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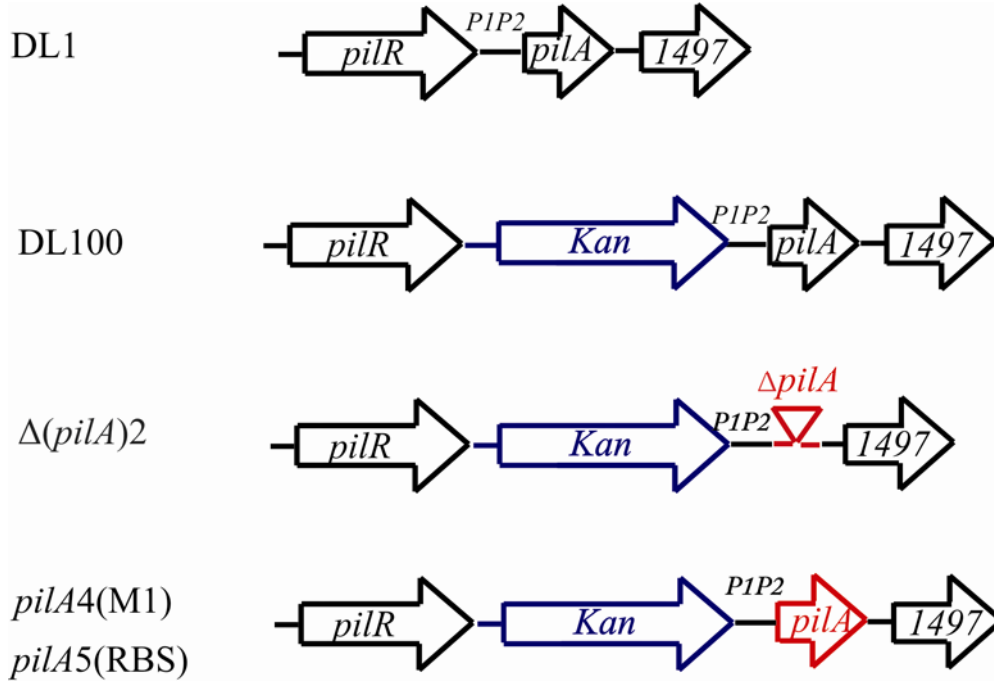
\* The intensity of the PilA bands from the wildtype DL100 strain appeared to be more than those of the PilA protein from the wildtype DL1 based on Western blot observations of samples run side by side; cultures grown at 25°C (*data not shown*).

To complement the mutation of the *pilA* gene in  $\Delta(pilA)2$ , the pLC3 plasmid was first utilized. pLC3 was constructed as described in Chapter 2. Briefly, the entire coding sequence of the *pilA* gene was cloned in the pLC1 vector, a derivative of the spectinomycin resistance-marked expression vector pRG5 (Kim et al., 2005) without the *tacUV5* & *lac* promoters (Chapter 2). Expression of the *pilA* gene from the pLC3 plasmid was regulated by its native promoters P1 and P2 (upstream of the *pilA* gene) to avoid overexpression. Western blot analysis using the PilA-specific antibody indicated that the full-length *pilA* gene together with its native promoter region on a plasmid could only restore expression of PilA but not secretion (Figure 29). However, analysis of the distribution of outer surface *c*-type cytochromes in the  $\Delta(pilA)2$ \_pLC3 strain revealed proper localization of OmcS and OmcZ at levels comparable to those of the wildtype (Figure 30). In the case of the pRG5\_*pilA* plasmid constructed previously (Reguera et al., 2005), a 5' truncation of the *pilA* gene (starting from the ATG codon) that resulted in the loss of the long preprotein isoform was cloned in the pRG5 vector and *pilA* expression was regulated by the *tacUV5* & *lac* promoters. Expression of the *pilA* gene from the pRG5\_*pilA* plasmid in the  $\Delta(pilA)1$  strain (the strain engineered by Reguera *et. al.* 2005) resulted in outer surface localization of OmcZ; yet no OmcS was detected on the cells' outer surface\* (Figure 30).

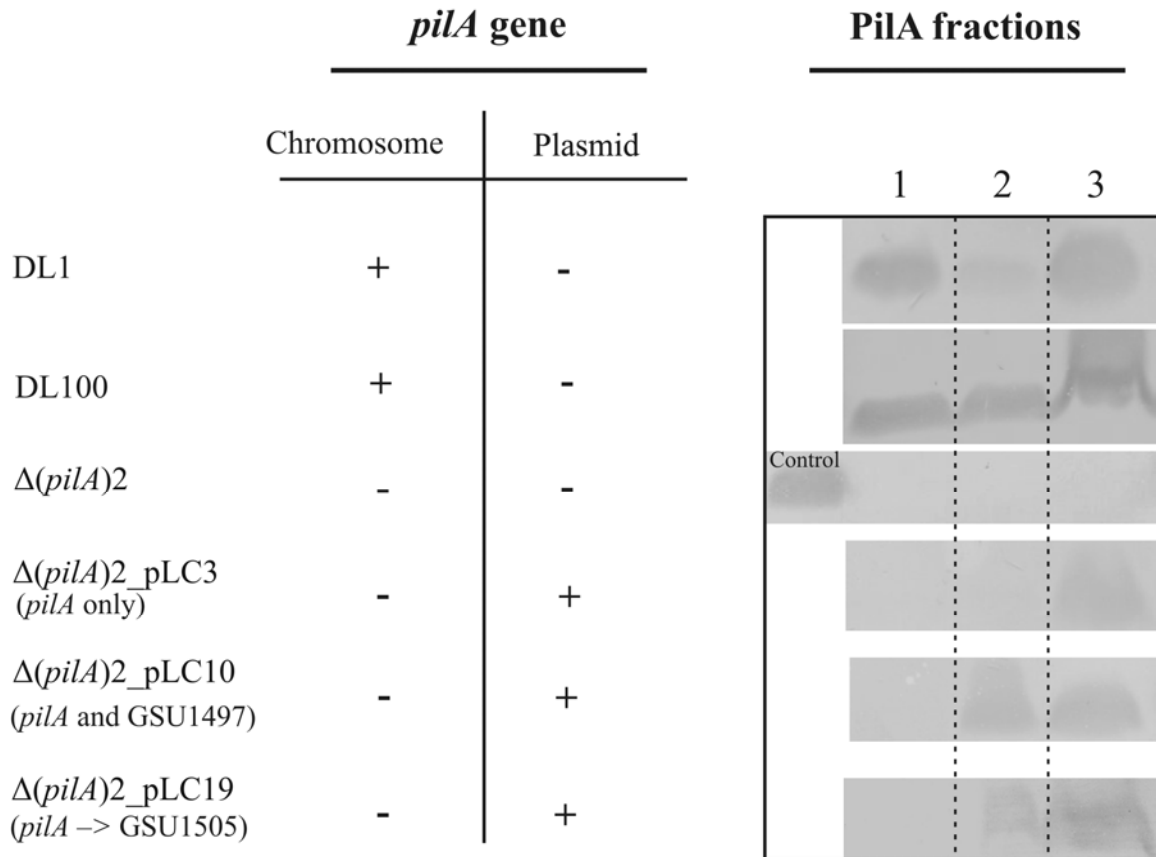
The data demonstrate a requirement for the entire coding sequence of the *pilA* gene (starting from the translation start codon TTG) in addition to the native promoters region upstream in order to re-establish a proper localization of the *c*-type cytochromes in comparable levels to the wildtype.

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\* Izallalen and Lovley - Unpublished observations.



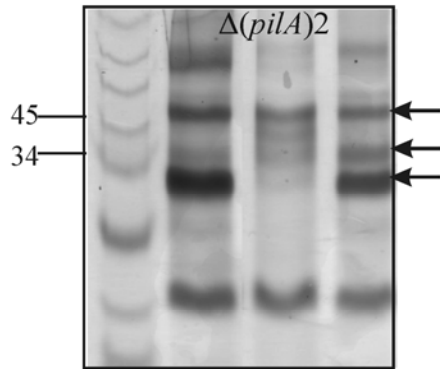
**Figure 28.** Illustration of gene organization in the mutant strains used in Chapter 4. The kanamycin resistance cassette was inserted on the 5' side of the P1 and P2 promoter region in all the constructed strains. DL1 is wildtype, DL100 is an isogenic wildtype *pilA* control with a kanamycin resistance cassette in before the promoter region,  $\Delta(pilA)2$  is the *pilA* in-frame deletion mutant strain, *pilA4*(M1) is the short isoform strain that harbors the translation start codon TTG→CCC mutation, and *pilA5*(RBS) is the long isoform strain that harbors ribosome-binding site mutations. The *pilA* gene is depicted in black for wildtype and in red when it carries mutation(s).



**Figure 29.** Expression of the *pilA* gene from different plasmids in the  $\Delta(pilA)2$  mutant strain. The subcellular fractions are (1) supernatant fraction, (2) cell-associated fraction 1 and (3) cell-associated fraction 2. All PilA protein fractions of samples collected from the wild type (DL1 & DL100) and the studied mutant strains migrated at 7 kDa.

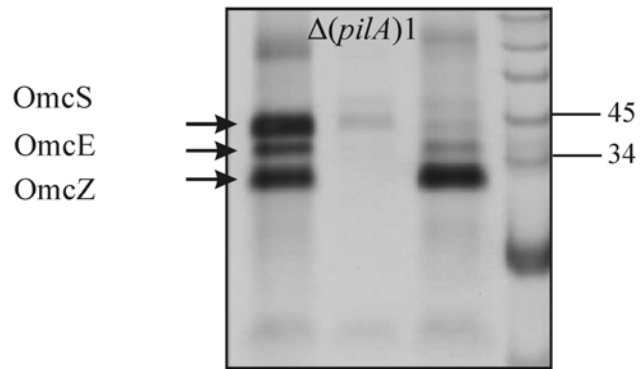
A

	<u><i>pilA</i> gene</u>		
Plasmid	-	-	+ (pLC3)
Chromosome	+	-	-



B

	<u><i>pilA</i> gene</u>		
Plasmid	-	-	+ (pRG5 w/ <u>truncated</u> <i>pilA</i> )
Chromosome	+	-	-



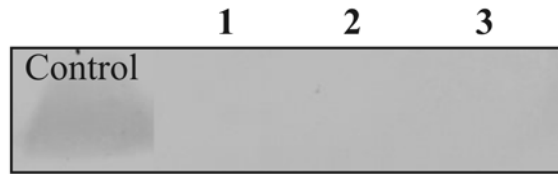
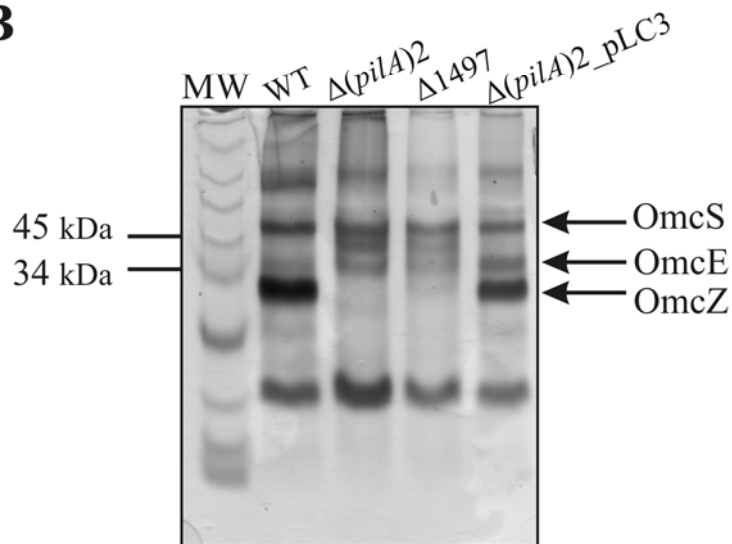
Izallalen and Lovley - Unpublished observations

**Figure 30.** Comparison of outer membrane *c*-type cytochrome distribution when the entire *pilA* vs. an N-terminally truncated sequence of *pilA* is expressed. (A) The composition of outer membrane *c*-type cytochromes in the in-frame *pilA* deletion mutant and complemented strain constructed for this study vs. (B) the *pilA* deletion mutant and complemented strain (with a 5' truncation of *pilA* that resulted in the loss of the long isoform preprotein) used by Reguera *et al.* 2005.  $\Delta(pilA)1$  is the knockout mutant constructed and used by Reguera *et al.* (Reguera *et al.*, 2005),  $\Delta(pilA)2$  is the in-frame *pilA* deletion mutant constructed as described in Chapter 2.

## Investigation of the role of GSU1497 and the 3' end gene cluster in expression and secretion of PilA

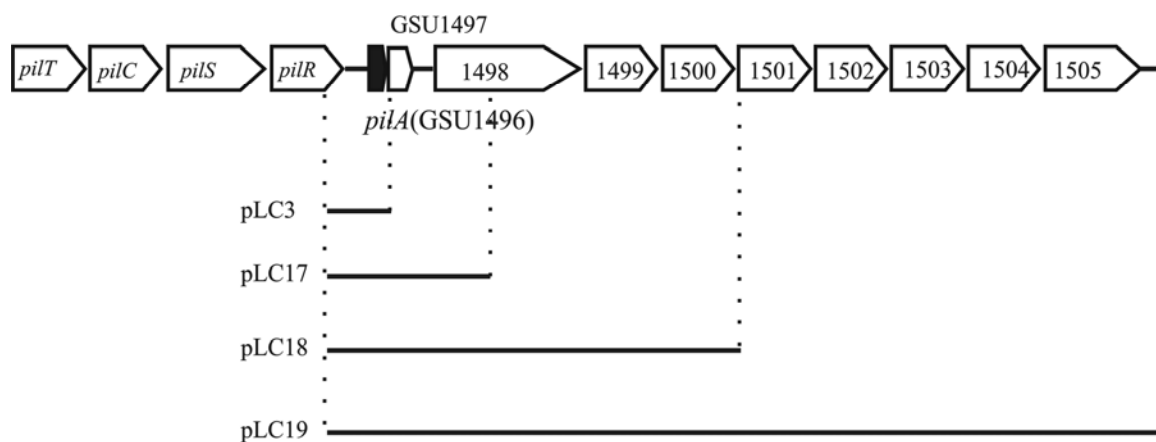
Due to the fact that expression of the full-length *pilA* gene in *trans* did not restore secretion of the PilA protein in the  $\Delta(pilA)2$  strain, an investigation of the genes at the 3' end of *pilA* was conducted. A thorough study was carried out on GSU1497 due to the availability of the strain in which GSU1497 was deleted (Nevin et al., 2009). GSU1497 is a gene of 375 bp that is co-transcribed with the *pilA* gene (GSU1496); no data are available on the GSU1497 protein's function. Western blot analysis of protein samples prepared from the  $\Delta$ GSU1497 strain and probed with the PilA-specific antibody revealed an interesting finding. Although the transcription level of the *pilA* gene in the  $\Delta$ GSU1497 strain is comparable to that of the wildtype strain (Dr. A. Klimes, *personal communication*), no PilA protein was detected in either the intracellular or the extracellular fractions of  $\Delta$ GSU1497 cells (Figure 31A). Additionally, mislocalization of the outer surface *c*-type cytochromes was observed by heme-stained SDS-PAGE (Figure 31B), which seems to be a consistent finding in cells lacking PilA (compare Figure 30). In addition to these findings, it was recently reported that the  $\Delta$ GSU1497 strain produced a maximum current of magnitude two-thirds that of the wildtype, and took five times longer to reach maximum current production (Nevin et al., 2009). The observations indicate that GSU1497 is absolutely required to stabilize PilA; therefore, the plasmid pLC10, harboring both *pilA* and GSU1497, was tested next as a strategy to restore secretion of PilA. Co-expression of *pilA* and GSU1497 in the  $\Delta(pilA)2$  strain from a plasmid (pLC10, Figure 32), in which *pilA* was under the control of its native promoters increased the amount of intracellular PilA protein in the soluble fraction, but did not restore secretion of PilA (see Figure 29 for the Western blot).



**A****B**

**Figure 31.** Characterization of the  $\Delta$ GSU1497 strain. (A) Western blot analysis of protein samples prepared from the  $\Delta$ GSU1497 strain and probed with the PilA-specific antibody. The subcellular fractions are (1) supernatant fraction, (2) cell-associated fraction 1 and (3) cell-associated fraction 2. (B) Heme-stained SDS-PAGE of the loosely bound outer surface *c*-type cytochromes. Each lane contains 3  $\mu$ g of protein.

Microarray analysis data collected in the Lovley lab for cells grown under various conditions (Table 9) revealed a similar expression pattern for the *pilA* gene, GSU1497, and a gene cluster on its 3' side. This gene cluster (GSU1498 to GSU1505) encodes ABC-type transporters, cell wall glycosylation enzymes, and other proteins of unknown function (Krushkal et al., 2010). A homolog gene cluster, with several ABC transporter proteins, located downstream of the type IV pilin gene in *M. xanthus* was reported to be involved in PilA protein secretion (Wu et al., 1998). We tested the hypothesis that to have the gene cluster co-expressed with *pilA* and GSU1497 in *cis* was important for secretion of PilA. The pLC19 plasmid was constructed for that purpose. Expression of the *pilA* gene from pLC19 is regulated by its native promoters and the only difference in the pLC19 plasmid *versus* the pLC3 and pLC10 plasmids is the addition of nine genes at the 3' end of GSU1496 (Figure 32). No secreted PilA was detected by Western blot for the  $\Delta(pilA)2\_pLC19$  strain. The *pilA* deletion mutant strain was constructed with caution to avoid a polar effect on the downstream genes, and the sequence around the deleted *pilA* region was confirmed by DNA genomic sequencing. Nevertheless, secretion of PilA could not be restored with any of the constructed plasmids; the data did not indicate that the deletion caused a polar effect on any of the 3' side genes, nor that co-expression of the genes was significant. Further investigation is required to understand why full complementation is not achieved, and to test whether the DNA segment deleted from the chromosomal *pilA* in the  $\Delta(pilA)2$  strain is important for other regulatory processes (e.g. RNA secondary structure).



**Figure 32.** Illustration of the DNA sequence cloned in each of the plasmids used for complementation in this work. The *pilA* gene is darkened.

<b>Microarray experimental conditions</b>	<b>GSU1496 (<i>pilA</i>)</b>	<b>GSU1497</b>	<b>GSU1498→ GSU1505</b>
Differential gene expression of <i>G. sulfurreducens</i> biofilms on Fe(III) oxide vs. planktonic cells	Downregulated	Downregulated	Downregulated
Fe(III) citrate, <i>G. sulfurreducens</i> DL1 (WT) compared to DL16 ( <i>rpoS</i> sigma-38 factor mutant)	Upregulated	Upregulated	Upregulated
Transcriptional regulator FNR1 knockout vs. PCA grown on fumarate	Upregulated	Upregulated	Upregulated
Transcriptional regulator FNR2 knockout vs. PCA grown on fumarate	Upregulated	Upregulated	Upregulated
Knockout of acetate kinase and phosphate acetyltransferase vs. WT	Upregulated	Upregulated	Upregulated
Transcriptional regulator <i>pilR</i> mutant (GSU1495) vs. WT	Downregulated	Downregulated	Downregulated
Transcriptional regulator IdeR mutant vs. WT	Upregulated	Upregulated	Upregulated
Transcriptional regulators Fur & IdeR (dtx) double mutant vs. Wildtype	Upregulated	Upregulated	Upregulated
Fe gel vs. Fe(III) citrate	Upregulated	Upregulated	Upregulated

**Table 9.** Comparison of the transcriptional levels of *pilA*, GSU1497 and the 3' end gene cluster under various growth conditions (*Geobacter*).

### **Complementation of the *pilA4*(M1) and *pilA5*(RBS) phenotypes by expression of the *pilA* gene in *trans***

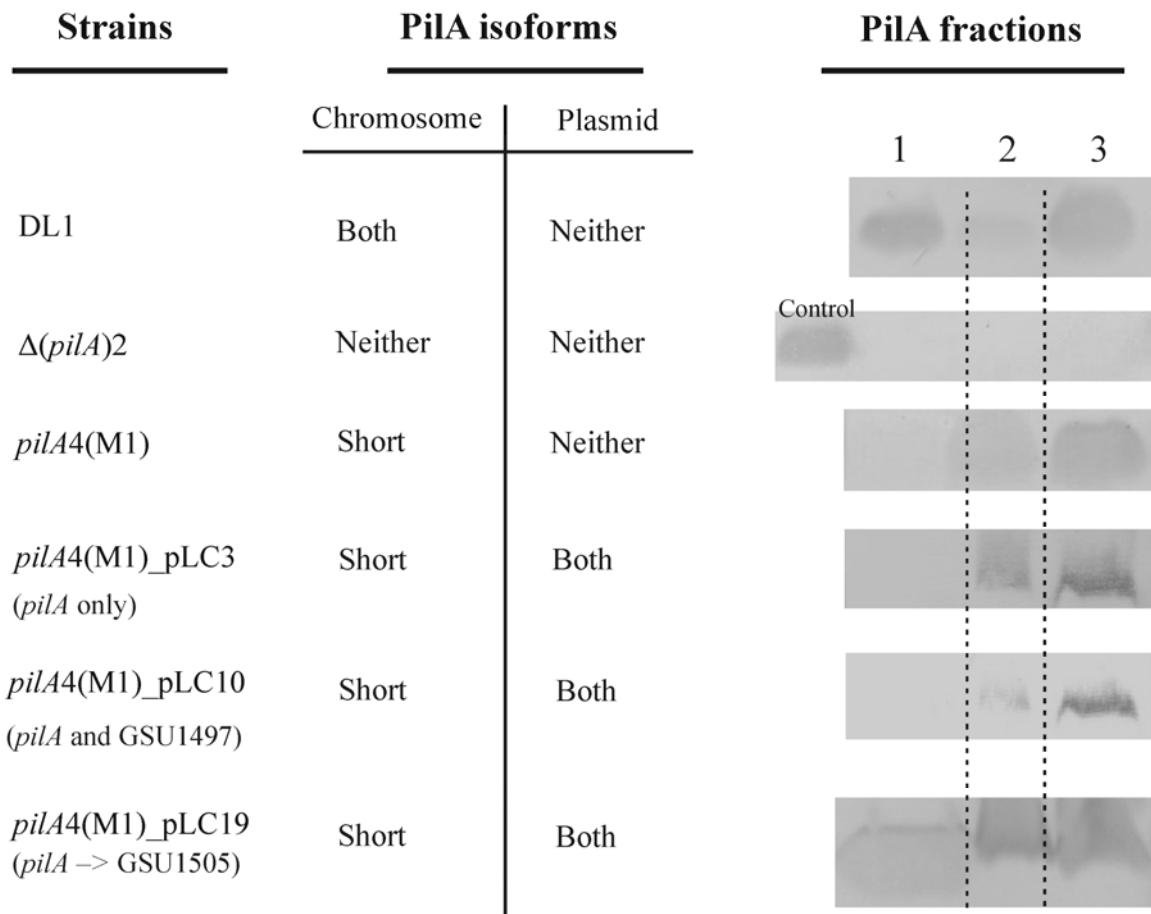
The pLC3, pLC10 and pLC19 plasmids (Figure 32) were each used to express the *pilA* gene in *trans* in the *pilA4*(M1) and *pilA5*(RBS) mutant strains. The short isoform *pilA4*(M1) strain has mutations in the translation start codon of the long PilA preprotein isoform, whereas the long isoform *pilA5*(RBS) has mutations in the ribosome-binding site of the short PilA preprotein isoform (Chapter 3). Western blot analysis of protein fractions prepared from the *pilA4*(M1) strain indicated the absence of extracellular PilA, but PilA was still detected intracellularly (Figure 33, also see Chapter 3). However, no PilA protein was detected by Western blot for any of the cell fractions when the short PilA isoform was eliminated in *pilA5*(RBS) (Figure 34, also see Chapter 3).

The pLC3 plasmid contains the entire coding sequence of the *pilA* gene under the control of the native P1 and P2 promoters. Expression of the *pilA* gene from the pLC3 plasmid restored the intracellular soluble and membrane-associated fractions of the PilA protein in the *pilA5*(RBS) strain (Figure 34). However, no secreted protein was detected for either the *pilA5*(RBS) or the *pilA4*(M1) strain complemented with pLC3 (Figures 33 & 34). The data suggest that a copy of the entire *pilA* gene on a plasmid is sufficient to restore expression of the PilA protein but not its secretion. Similar observations were noted when *pilA* was co-expressed with GSU1497 from the pLC10 plasmid; the PilA protein was detected intracellularly, yet no protein was detected extracellularly for any of the complemented strains (Figures 33 & 34).



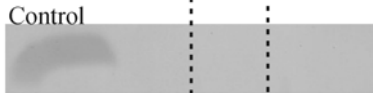

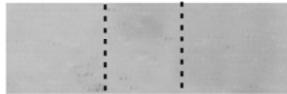
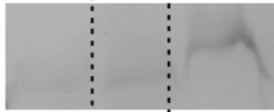
The secreted PilA protein was detected by Western blots of samples prepared from the *pilA4*(M1)\_pLC19 and *pilA5*(RBS)\_pLC19 strains (Figures 33 & 34). These data indicate a requirement for co-expression of the *pilA* gene, GSU1497 and one or more

of the other eight genes (GSU1498 → GSU1505) in *trans* in order to achieve full complementation (expression and secretion) of either of the single-isoform *pilA* mutant strains.

To verify that the secreted PilA protein detected by Western blot was functional, assays of attachment to glass surfaces were performed for the *pilA4*(M1)\_pLC19 strain. Data obtained from the *pilA4*(M1) strain, in which intracellular soluble PilA and membrane-bound PilA were present, demonstrated that the absence of the extracellular PilA severely diminished the ability to attach to glass surfaces in the presence of soluble electron donor and acceptor (Chapter 3). Similar attachment assays (performed as described in Chapter 3) were conducted on the *pilA4*(M1)\_pLC19 strain. The reappearance of extracellular PilA was accompanied by an enhanced ability to attach to glass surfaces to an extent typical of the wildtype (Figure 35). Thus, for the first time, full complementation of a loss-of-function mutation of *pilA* has been demonstrated: as long as one of the isoforms is expressed from the native context, and the other isoform is co-expressed with genes from GSU1497 to GSU1505, they can co-operate to achieve secretion of functional PilA. In the case of the  $\Delta(pilA)2$  strain, expression of *pilA* from the pLC19 plasmid did not result in secretion of PilA; possibly because of higher order function of the deleted DNA segment of chromosomal *pilA*. To confirm this hypothesis, the expression of *pilA* should be tested in the *pilA7* strain, in which both start codons are altered, with the pLC19 plasmid. If our hypothesis is correct, then full complementation and secretion will be achieved. If not, then this implies a requirement for having at least one isoform with more context than the nine 3' side genes.

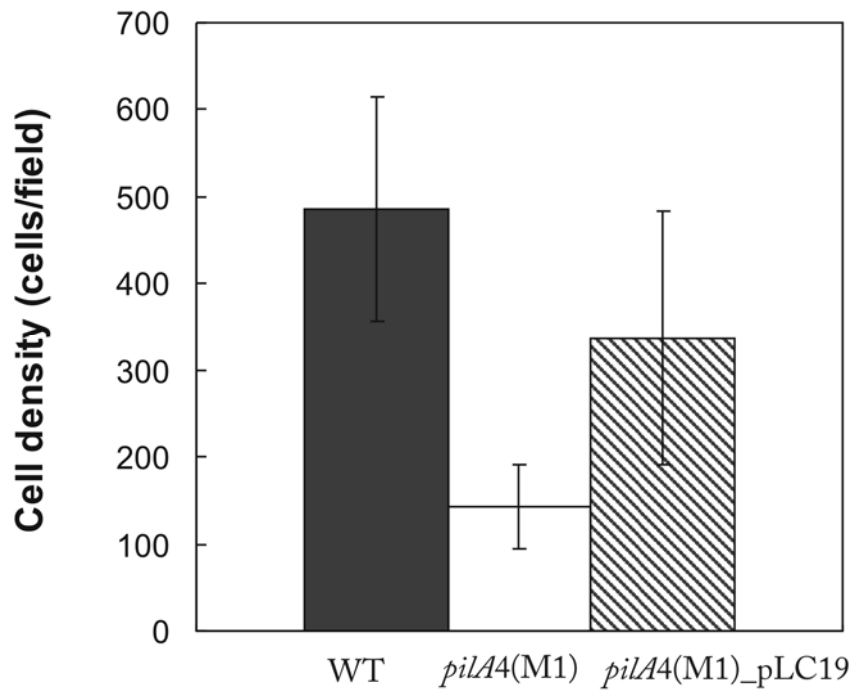


**Figure 33.** Expression of the *pilA* gene from different plasmids in the *pilA4*(M1) mutant strain. The PilA-specific antibody was used in all Western blots. The subcellular fractions are (1) supernatant fraction, (2) cell-associated fraction 1 and (3) cell-associated fraction 2. All PilA proteins of samples collected from the wild type DL1 and the studied mutant strains were detected at 7 kDa.

Strain	PilA isoforms		PilA fractions		
	Chromosome	Plasmid	1	2	3
DL1	Both	Neither			
$\Delta(pilA)2$	Neither	Neither			
<i>pilA5</i> (RBS)	Long	Neither			
<i>pilA5</i> (RBS)_pLC3 ( <i>pilA</i> only)	Long	Both			
<i>PilA5</i> (RBS)_pLC10 ( <i>pilA</i> and GSU1497)	Long	Both			
<i>pilA5</i> (RBS)_pLC19 ( <i>pilA</i> → GSU1505)	Long	Both			

**Figure 34.** Expression of the *pilA* gene from different plasmids in the *pilA5*(RBS) mutant strain. The PilA-specific antibody was used in all Western blots. The subcellular fractions are (1) supernatant fraction, (2) cell-associated fraction 1 and (3) cell-associated fraction 2. All PilA proteins of samples collected from the wild type DL1 and the studied mutant strains were detected at 7 kDa.





**Figure 35.** Attachment assay of the *pilA4*(M1)\_pLC19 strain to glass surfaces. Bacterial strains are the wildtype (solid bar), the *pilA4*(M1) mutant (open bar) and the *pilA4*(M1)\_pLC19 complemented strain (shaded bar). Cells were incubated for four days on glass surfaces with freshwater fumarate-acetate media at 25°C under strictly anaerobic conditions. Data are the average of six biological replicates from two independent experiments. Error bars represent one standard deviation.

## **Summary and conclusions**

The type IV pili of *Geobacter sulfurreducens* were previously proposed to be electrically conductive and to mediate long-range electron transfer to Fe(III) oxide and graphite electrodes. Deletion of *pilA*, the gene for the structural protein PilA, caused reduced levels of Fe(III) oxide reduction and current production. However, subsequent studies demonstrated that the *pilA* deletion mutant not only lacks the PilA protein but does not properly localize several outer surface *c*-type cytochromes that are known to be essential for optimal Fe(III) oxide reduction and current production. A genetically engineered complemented strain restored partial Fe(III) oxide reduction and wildtype-level current production. However, outer surface *c*-type cytochromes were still not properly localized.

Sequence analysis of the previously engineered PilA expression plasmid indicated a clone of a 5' truncated sequence of the *pilA* gene with only the internal translation start codon (ATG). Because our data demonstrated the presence of two functional translation start codons for the *pilA* gene (TTG and ATG) (Chapter 3), we constructed the expression plasmids for our study of complementation so that the entire *pilA* gene (starting from the external start codon TTG) was under the control of its native promoters.

Complementation of several *pilA* chromosomal mutant strains was tested with plasmids containing *pilA* alone, *pilA* plus GSU1497, or *pilA* along with the nine genes on its 3' side that share its pattern of differential expression. The key genes required for full complementation, i.e. both expression and secretion, were determined to be between GSU1496 and GSU1505. The summary of our results is as follows:

- Expression of the full-length *pilA* gene from its native promoters restores some intracellular PilA protein production, but it is not secreted.
- The intracellular PilA protein is sufficient for secretion of the outer surface *c*-type cytochromes at levels comparable to the wildtype.
- Co-expression of *pilA*, GSU1497 and a downstream gene cluster (GSU1498 to GSU1505, encoding ABC transport system components and glycosylation enzymes) restored the expression and secretion of PilA in strains that expressed only one isoform from the *pilA* gene on the chromosome, but not in a strain bearing a chromosomal deletion of *pilA*. A process of similar complexity has been documented in the formation of type IV pili filament bundles in EPEC (Stone et al., 1996).

A recent report indicated that co-expression genes GSU1500-1505 together, *in trans*, was required to complement the chromosomal deletion of the GSU1501 gene (Rollefson et al., 2010). The GSU1501 deletion strain lacked the extracellular polysaccharide matrix, and co-expression of the entire gene cluster (GSU1500-1505) from a plasmid was necessary to restore the polysaccharide network and to produce partial-wildtype-level phenotypes in surface attachment and insoluble Fe(III) reduction.

- The reappearance of extracellular PilA protein due to co-operation between the long and short isoforms expressed from different loci restored the cells' ability to attach to glass surfaces similarly to the wildtype.

## CHAPTER 5

### FUTURE STUDIES

#### **Amino acids important for conductivity and Fe(III) oxide reduction in the C-terminal portion of PilA**

In Chapter 2, we identified a non-conserved tyrosine located in the C-terminal segment of the PilA protein that is essential for biofilm formation and for cell growth on Fe(III) oxide. We altered this amino acid by introducing a point mutation via site-directed mutagenesis and demonstrated that this did not affect expression or secretion of the protein under the applied experimental conditions. Furthermore, we demonstrated that insertion of the kanamycin resistance marker before the P1 and P2 promoter region of *pilA* did not cause a frame shift or polar effect regarding the expression or secretion of PilA.

Therefore, we propose to use the same approach used in Chapter 2 to investigate further the amino acids of the C-terminal segment of PilA in order to elucidate the electron transfer pathway along the nanowires.

#### **Hypothesis**

The C-terminal region of PilA harbors amino acids that are important for PilA function; and amino acid substitution of any of these residues would affect the Pili function without disrupting the pilus assembly.

#### **Supporting Observations**

- The N-terminal segment of all type IV pilins is a highly conserved hydrophobic helix that forms the core of the pilus filament. However, the C-terminal region has

a sequence that varies between species, depending on the protein's function (Mattick, 2002).

- The C-terminal segments of all type IV pilins whose crystal structures are solved contain regions with charged amino acids that are exposed to the outside environment (Parge et al., 1995; Craig et al., 2004; Xu et al., 2004; Craig et al., 2006).
- Since the phenomenon of electrically conductive type IV pili has been reported in *Geobacter* species exclusively (Reguera et al., 2005); the non-conserved C-terminal segment is expected to contain the molecular features responsible for electron transfer through the nanowires.
- Our study (Chapter 2) demonstrated that a non-conserved tyrosine in the C-terminal segment of the mature PilA is essential for biofilm formation and for cell growth on Fe(III) oxide.
- Cells with a tyrosine-32 to phenylalanine point mutation were unable to grow with Fe(III) oxide, a phenotype that was reported for the PilA-deficient strain (Reguera et al., 2005) (Chapter 2).
- Charged/aromatic amino acids in the C-terminal segment may contribute to the pili function and site-directed mutagenesis is a feasible technique to test this hypothesis (Kovacic, 2007).

## **Approach**

- Site-directed mutagenesis will be utilized to introduce a point mutation in the *pilA* gene on a plasmid. The choice of plasmid and a full explanation of the method are presented in Chapter 2.

- The point mutation will then be introduced into the chromosomal copy of *pilA* by recombinant PCR and a drug resistance marker will be inserted on the 5' side of the P1 and the P2 promoter region as described in Chapter 2. An isogenic control with the drug resistance marker in front of the wildtype *pilA* gene will also be isolated and tested for PilA expression and secretion.
- Screening for phenotypes:
  - Expression and secretion of PilA in the mutant strain will be checked by fractionating the cell culture to isolate the intracellular and extracellular protein fractions as described in Chapter 2. The PilA-specific antibody will be used to detect the PilA protein in each fraction by Western blot.
  - The expression and proper distribution of outer surface and outer membrane cytochromes will be checked by means of Western blotting and heme staining of SDS PAGE.
  - The performance of the mutant strain in microbial fuel cells will be compared to the wildtype. This experiment will determine whether the amino acid mutated is important for electron transfer to graphite electrodes.
  - The cells' ability to grow on insoluble Fe(III) oxide will be tested to determine whether the mutated amino acid is part of the electron transfer pathway to insoluble metal oxide.

## **Investigation of the role of GSU1497 in electron transfer to insoluble electron acceptors**

### **Hypothesis**

GSU1497 forms a protein complex with GSU1496 (PilA) by binding to its C-terminus, and the electron transfer pathway extends from PilA over GSU1497 to the insoluble electron acceptor.

### **Supporting Observations**

- GSU1497 is a small gene (375 bp) encoding a hypothetical protein with no clear function.
- The *pilA* and GSU1497 genes are predicted to form one operon and their co-transcription was confirmed experimentally (Figure 36).
- Deletion of the GSU1497 gene eliminated the PilA protein biogenesis (production and processing); subsequently, some *c*-type cytochromes were not detected on the cells' outer surface (Chapter 4).
- The  $\Delta$ GSU1497 mutant strain produced less current in microbial fuel cells and required 5 times longer to achieve maximum current (Nevin et al., 2009).
- In a model of pilus assembly (Craig et al., 2006), the hydrophobic helix of the mature pilin protein is packed in the core of the pilus fiber with the C-terminal domain folded into a four-stranded antiparallel  $\beta$ -sheet (termed the globular head) shielding the hydrophobic core from the outside environment. In the case of PilA of *G. sulfurreducens* the sequence of the mature protein is very short (61 amino acids vs. ~140 amino acids in other type IV pilins), and based on the sequence alignment to other type IV pilin proteins from Gram-negative bacteria (Appendix

D), the hydrophobic helix encompasses 40% of the protein sequence with a very small sequence for the C-terminal domain that is predicted to fold into a small extended coil (Figure 37). Therefore, and according to the model of pilus assembly, the hydrophobic helix would be exposed to the outside environment, which is energetically unfavorable.

- The secondary structure of GSU1497 was predicted with I-TASSER software and resembles that of the globular head of type IV pilins from other bacteria whose PilA proteins consist of a minimum of 140 amino acids. GSU1496 and GSU1497 together would make a protein complex that is comparable to PilA proteins of other bacteria in size and secondary structure.
- A polyclonal PilA antibody that was generated against the C-terminal segment of PilA does not recognize the native protein, although the strategy used to generate the antibody is commonly applied to homologous type IV pili and the antibodies produced are usually reactive with the native proteins (Li et al., 2005). Therefore, it is possible that the C-terminal domain of the PilA protein is blocked by another molecule or fused protein (possibly GSU1497) that prevents the access of the antibody to the PilA protein.

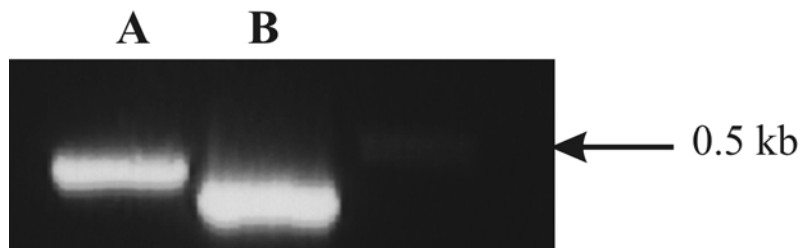
### **Approach**

GSU1497 will be overexpressed in *E. coli*. The theoretical molecular mass for the protein is 8.93 kDa (EXPASY, Expert Protein Analysis System). The overproduced protein will be used to generate a GSU1497-specific antibody that is expected to be reactive with the native protein.

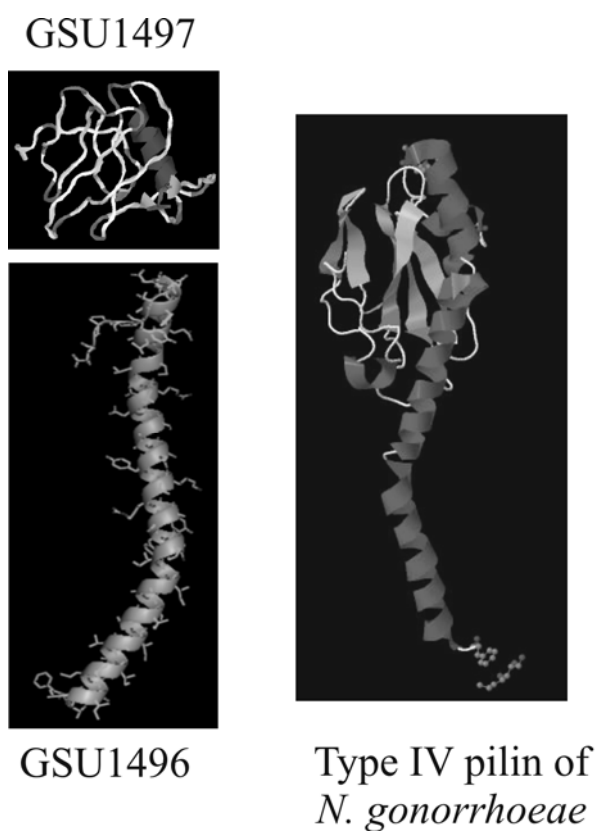


Immunogold-labeled GSU1497 antibody will be used with *G. sulfurreducens* wildtype cells (DL1) to check whether the protein is detected extracellularly and along any filamentous structures. The *pilA3*(M1) strain (Chapter 3) would be used as a negative control. This strain produces intracellular PilA only, and so no extracellular filamentous PilA is expected to be present. Since *pilA* and GSU1497 are co-transcribed, transcription of GSU1497 should not be affected in this strain because the point mutation occurs in the translation start codon of the long PilA isoform and was shown not to affect transcription of *pilA* (Chapter 3).

If our hypothesis turns out to be correct then point mutations could be introduced in redox-active amino acids of GSU1497 that are exposed to the surface (Figure 38) and the mutant strains would be checked for the ability to grow on insoluble electron acceptors (graphite anodes and Fe(III) oxide).



**Figure 36.** DNA gel of the qualitative reverse transcriptase PCR reactions of  $\Delta(pilA)2\_pLC3$ . Both reactions were conducted on cDNA generated from  $\Delta(pilA)2\_pLC3$  RNA as a template and using a set of primers designed to amplify (A) the *pilA* and GSU1497 genes together and (B) the GSU1497 gene alone. The DNA gel is made with 2% agarose. Expected band sizes are 486 bp for A and 402 bp for B.



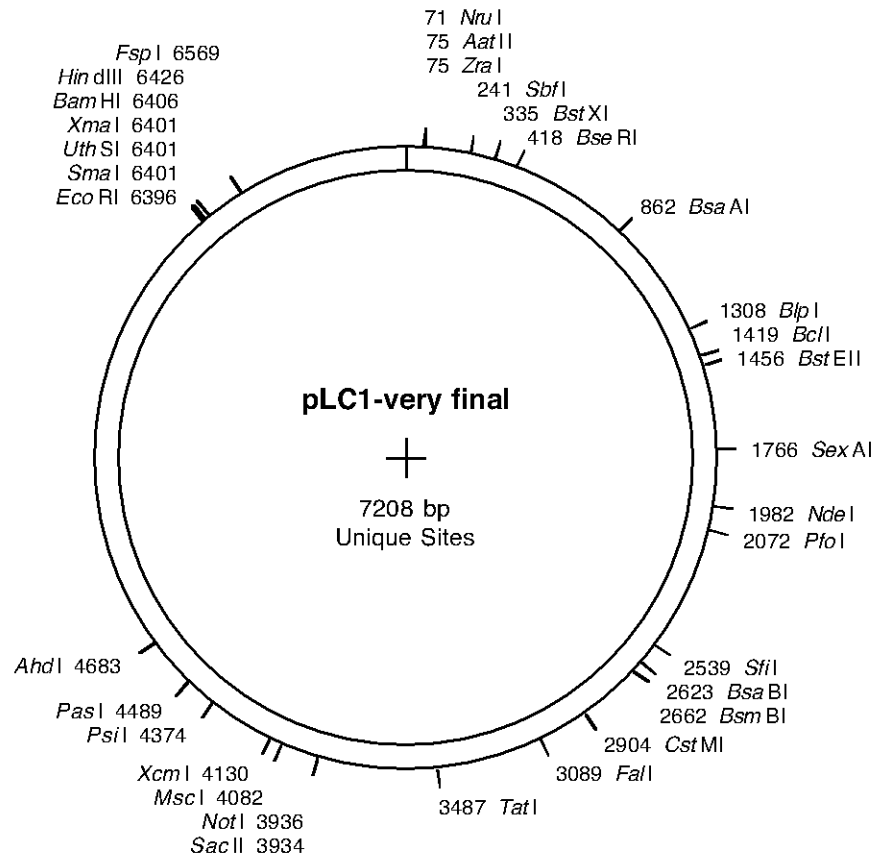
**Figure 37.** Predicted secondary structure of GSU1496 (PilA) and GSU1497 using I-TASSER (I-TASSER, Protein Structure & Function Predictions). Images are courtesy of Ned Young. The source of solved secondary structure of type IV pilin of *N. gonorrhoeae* is the Protein Data Bank (PDB ID 2HI2).

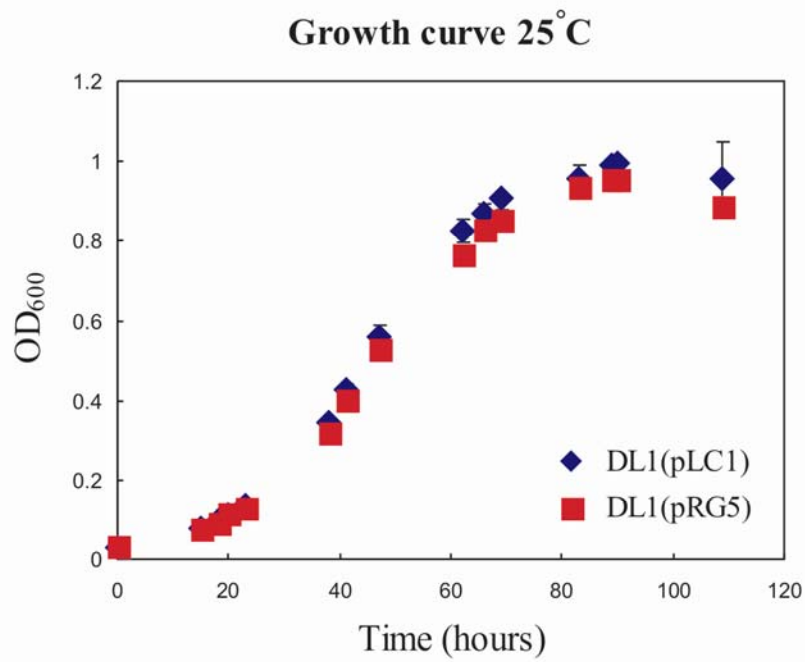
10 20 30 40 50 60  
 MKKIITIVAM LLAMQGIAIA AGKIPTTTGM GKDFTFKPST NVSVSYFTTN GATSTAGTVN  
 70 80 90 100 110 120  
 TDYAVNTKNS SGNRVFTSTN NTSNIWYIEN DAWKGKAVSD SDVTALGTGD VGKSDFSGTE  
 WKSQ

**Figure 38.** Amino acid sequence of the GSU1497 protein. Predicted surface-exposed residues are colored in blue (NetSurfP, Protein Surface Accessibility and Secondary Structure Predictions).

## APPENDIX A

### THE PLC1 PLASMID MAP, AND GROWTH CURVES OF DL1 HARBORING EITHER PRG5 OR PLC1





Minipreps for both plasmids gave comparable DNA yield

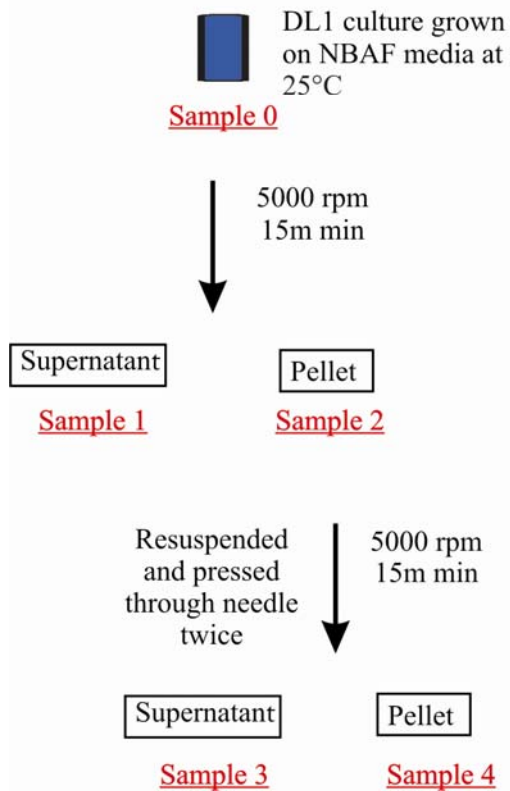


Both plasmids replicate with the same frequency in *G. sulfurreducens*

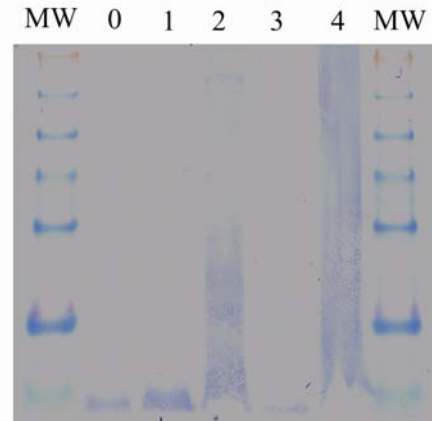
## APPENDIX B

### LOCALIZATION OF THE PILA PROTEIN IN CELL CULTURE

#### Sample preparation



#### Western blot



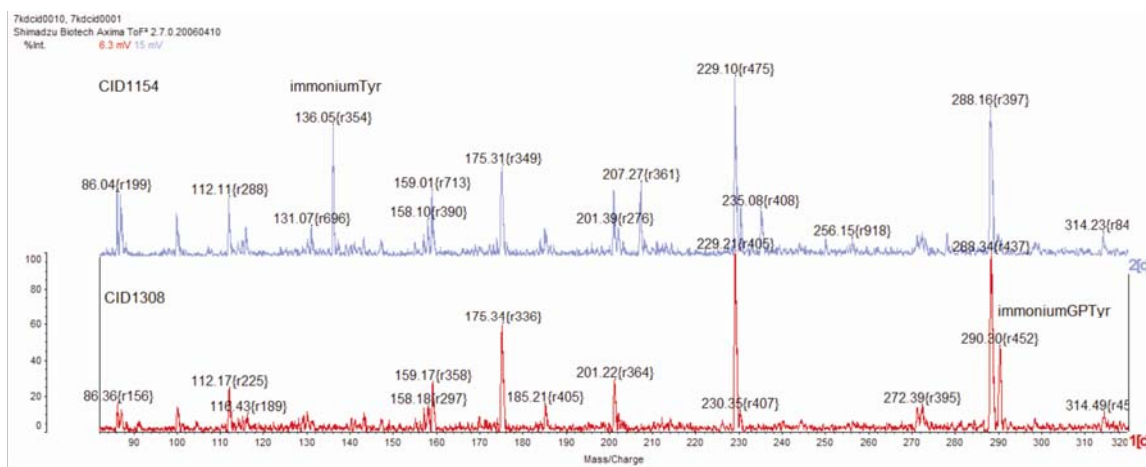
DL1 wildtype strain was grown in 10 mL Acetate-Fumarate (NBAF) media until the OD<sub>600</sub> equaled 0.6. 15 µL of the liquid culture was taken away (sample 0) and the remaining culture was harvested at 5000 rpm for 15 min. The supernatant was sampled (sample 1) and the pellets were resuspended in 2 mL of 100 mM Tris, pH= 7.5 (sample 2). Resuspended pellets were pressed through a needle twice (0.45 x 12 mm/26 G x ½ Luer-Lock Braun- Melsungen), then centrifuged at 5000 rpm for 15 min. The supernatant was sampled (sample 3) as well as the pellet (sample 4). All samples were 15 µL.

## APPENDIX C

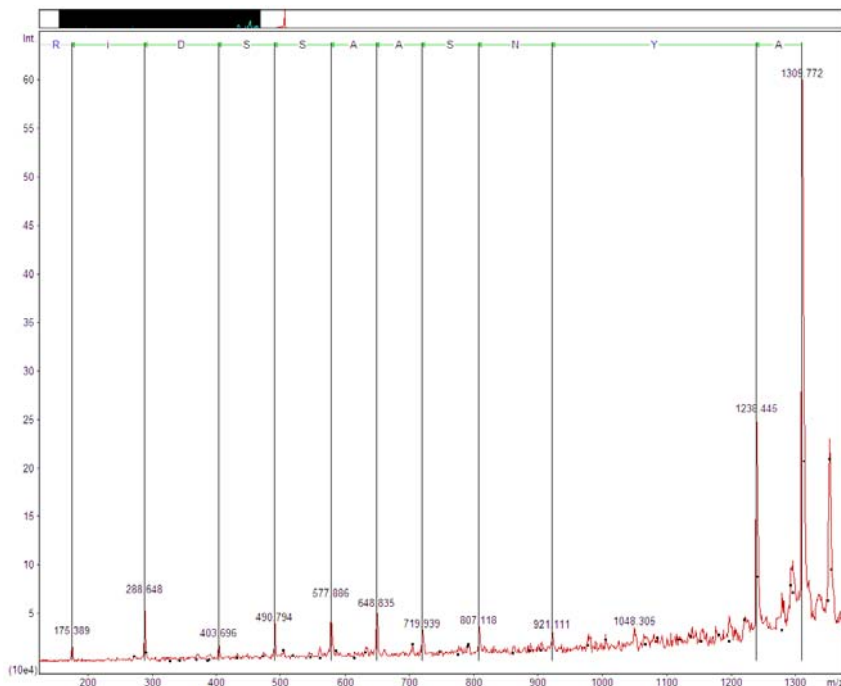
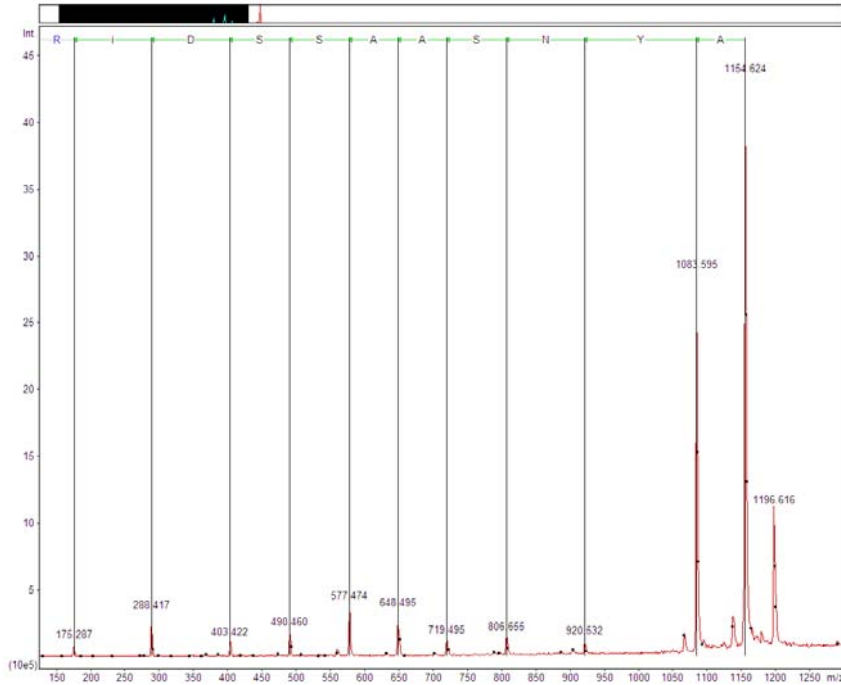
### MATRIX-ASSISTED LASER DESORPTION/IONIZATION (MALDI) MASS SPECTROMETRIC DATA OBSERVED FOR THE TRYPTIC DIGESTED PEPTIDE (AYNSAASSDLR) OF THE PILA PROTEIN SECRETED BY *G. SULFURREDUCTENS*

Analyses were carried out at the University of Massachusetts Medical School, Laboratory for Mass Spectrometry, Worcester, MA. (University of Massachusetts Medical School)

Comparison of the MALDI spectra of the unmodified (top) and modified (down) (AYNSAASSDLR) peptide obtained by Collision Induced Dissociation **CID**. The 1154 Da peptide displays the unmodified tyrosine residue at 136 Da, which is not visible in the 1308 Da peptide. The new peak emerging at 290 Da (in the 1308 Da peptide) corresponds to a mass difference of 154 Da, suggesting a glycerophosphate-modified tyrosine.



MALDI-**PSD** (Post- Source Decay) mass spectrometric spectra of the unmodified (top panel) and modified (down panel) (AYNSAASSDLR) peptide. The amino acid sequence was deduced from the spectrum based on the mass difference between adjacent peaks. The mass difference of 317 Da between the peaks at 1238.445 and 921.111 Da (low panel) corresponds to the combined mass of a tyrosine residue (163 Da, upper panel) and a glycerophosphate group (154 Da).



## APPENDIX D


### PROTEIN SEQUENCE ALIGNMENT OF GSU1496, TYPE IV PILIN OF *G. SULFURREDUCTENS*, AND OTHER TYPE IV PILINS FROM GRAM-NEGATIVE BACTERIA

CLUSTAL 2.0.12 multiple sequence alignment

		↓ ↓		
Geobacter sulfurreducens	MANYPHTPTQAAKRRKETLMLQKLR-NRKGF	TLIELLIVVAII	GILAAIA 49	
Geobacter lovleyi	-----MLNKIR-NRKGF	TLIELLIVVAII	GILAAVA 30	
Pelobacter carbinolicus DSM2380	-----MLKELRKNEKGFTLIELLIVVAII	GILAAIA 31		
Geobacter uraniireducens Rf4	-----MLNKLR-SNKGFTLIELLIVVAII	GILAAIA 30		
Geobacter FRC32	-----MLNKLR-NKEGFTLIELLIVVAII	GILAAIA 30		
Geobacter M18	-----MLNKIR-SNKGFTLIELLIVVAII	GILAAIA 30		
Geobacter metallireducens	-----MLQKLR-NKKGFTLIELLIVVAII	GILAAIA 30		
Geobacter bemidjensis	-----MLNKLR-SNKGFTLIELLIVVAII	GILAAIA 30		
Geobacter M21	-----MLNKLR-SNKGFTLIELLIVVAII	GILAAIA 30		
Pelobacter propionicus DSM2379	-----MLNKLR-NRKGF	TLIELLIVVAII	GILAAIA 30	
Myxococcus xanthus DK1622	-----MRVSRFNPNRNGFTLIELMIVVAII	GILAAIA 32		
Neisseria gonorrhoeae MS11	-----MSNKMEQKGF	TLIEMMIVVAIL	GIIISVIA 29	
Neisseria meningitides Z2491	-----MMSNKMEQKGF	TLIEMMIVVAIL	GIIISVIA 30	
Neisseria meningitides C311	-----MNTLQKGF	TLIELMIVVIAIV	GILAAVA 27	
Shewanella baltica OS185	-----MSSIIRIEKGF	TLIELMIVVAII	GILAAIA 30	
Shewanella ANA3	-----MSALR---	KGFTLIELMIAVAII	GILAAIA 27	
Shewanella MR4	-----MSALR---	KGFTLIELMIAVAII	GILAAIA 27	
Vibrio mimicus 603	-----MKAYKNK-----	QKGF	TLIELMIVVAVIGVLAIA 31	
Pseudomonas aeruginosa PAO1	-----MK-----	AQKGF	TLIELMIVVAII	GILAAIA 26
Pseudomonas syringae	-----MNV-----	QKGF	TLIELMIVVAVIGVLAIA 26	
Pseudomonas aeruginosa PAK	-----FTLIELMIVVAII	GILAAIA 20		
Pseudomonas aeruginosa Pal10594	-----MKAQKGF	TLIELMIVVAII	GILAAIA 26	
Pseudomonas aeruginosa pa5196	-----MKAQKGF	TLIELMIVVAII	GILAAVA 26	
Pseudomonas aeruginosa K122	-----MKAQKGF	TLIELMIVVAII	GILAAIA 27	
Acinetobacter calcoaceticus	-----MN-----	AQKGF	TLIELMIVVAII	GILAAIA 26
Acinetobacter baumannii AYE	-----MN-----	AQKGF	TLIELMIVVAII	GILAAIA 26
Acinetobacter baumannii AB900	-----MN-----	AQKGF	TLIELMIVVAII	GILAAIA 26
Acinetobacter baumannii ATCC1797	-----MN-----	AQKGF	TLIELMIVVAII	GILAAIA 26
Vibrio vulnificus	-----MMKKLNKTK-----	KQGF	TLIELMIVVAVIGVLAIA 33	
Desulfuromonas acetoxidans	-----MLKKFRKNQKGF	TLIELLIVVAII	GILAAIA 31	
gi119899644	-----MKKL-----	QKGF	TLIELMIVVAII	GILAAIA 27
gi218780954	-----MIRLNKRN--	QGGF	TLIELMIVVAVIGVLAIA 31	
gi94265867	-----MIKMLQKRNGQKGF	TLVELMIVVAII	GILAAIA 33	
gi226227929	-----MN--RTRKGF	TLIELLIVVVI	GILAAIA 27	
gi94265584	-----MKQLTAWRDGEKGF	TLVELMIVVAII	GILAAIA 33	
gi85860123	-----MLRKLNNKRGQKGF	TLIELMIVVAII	GILAAIA 33	
gi218779223	-----MMQKLTKK--	ESGF	TLIELMIVVIAII	GILAAIA 31
gi114776286	-----MKLN-NEKGF	TLIELMIVVAII	GILAAIA 28	
gi224369860	-----MFKRLREKAK-NSKGF	TLIELMIVVIAII	GILAAIA 34	
gi258595733	-----MLQRMHQK-GQGGF	TLVELMIVVAVIGVLAIA 32		
gi225199913	-----MNLPKPAERSREQGF	TLIELLIVVAII	GILAAIA 34	
gi257092941	-----MKKLQKGF	TLIELMIVVAII	GILAAIA 27	
gi256830926	-----MNRFRKNEKGF	TLVELLIVVAII	GILAAIA 30	
gi226225417	-----MASRRASKGF	TLIELLIVVVI	GILAAIA 29	
gi85860121	-----MKLTRK-GNKGFT	TLIELMIVVIAII	GILAAIA 30	
gi258406253	-----MQMSKRNERGF	TLIELLIVVAII	GILAAIA 30	
gi158520534	-----MLRKMRKVTLGTQGF	TLIELMIVVIAII	GILAAIA 35	
gi257092940	-----MKS-RRGF	FALIELMIVVAVIGVLAIA 26		
gi14573312	-----MK-----	AQKGF	TLIELMIVVAII	GILAAIA 26
gi126165560	-----MK-----	AQKGF	TLIELMIVVAII	GILAAIA 26
gi31096010	-----MK-----	AQKGF	TLIELMIVVAII	GILAAIA 26
gi21464531	-----MK-----	AQKGF	TLIELMIVVAVIGVLAIA 26	
gi21629638	-----MK-----	AQKGF	TLIELMIVVAII	GILAAIA 26
gi545130	-----MK-----	AQKGF	TLIELMIVVAVIGVLAIA 26	
gi21629635	-----MK-----	AQKGF	TLIELMIVVAII	GILAAIA 26
gi120365	-----MK-----	AQKGF	TLIELMIVVAVIGVLAIA 26	



gi218893619 -----MK-----AQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 26  
gi256832576 -----MIARVAKAMNEKEKGFTLIELLVVIIIGILAAIA 35  
gi262197337 -----MLSKLQSRQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 31  
gi51245307 -----MKKALKNQQGF<sup>T</sup>LIELMIVVAIIIGILAAIA 30  
gil88591293 -----MQRVQQLKKLGRRVQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 37  
gil13866578 -----MQRVQQLKKLGRRVQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 37  
gil4573309 -----MK-----AQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 26  
gi239813460 -----MKRSITRAAQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 32  
gil49376813 -----MKSIQMNAQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 32  
gil4573315 -----MK-----AQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 26  
gi121534459 -----MLRNM<sup>R</sup>KAMKQKGF<sup>T</sup>LVELMVVAIIIGILAAIA 34  
gil51480 -----MK-----AQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 26  
gil20438 -----MK-----AQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 26  
gi88859017 -----MKQVKQMK---QQAQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 34  
gil82625519 -----MSIRSERRKKGF<sup>T</sup>LIELIIVVAIIIGILAAIA 31  
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gil10800846 -----MKKVSIRSKRRKKGF<sup>T</sup>LIELIIVVAIIIGILAAIA 34  
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gil4573303 -----MK-----AQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 26  
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gi49075736 -----MK-----AQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 26  
gil51463 -----STEIFMK-----AQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 31  
gil20405 -----MK-----AQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 26  
gi73540239 -----MQRVQ<sup>Q</sup>IKKLGR<sup>R</sup>VQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 37  
gil96158030 -----MMNMNTKNQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 31  
gi39704 -----MKS-----LQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 27  
gil20390 -----MKS-----LQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 27  
gil20383 -----MKS-----LQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 27  
gil20388 -----MKS-----LQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 27  
gil88996934 ---MERIFKSTAEAMSLTAKIEGERRKEKGF<sup>T</sup>LIELLIVIAIIIGILASIA 47  
gi92114295 -----MER-----QQRGF<sup>T</sup>LIELMIVVAIIIGILAAIA 27  
gi51245304 -----MKKALKNQQGF<sup>T</sup>LIELMIVVAIIIGILAAIA 30  
gil14331818 -----MQH-----NQQGF<sup>T</sup>LIELMIVVAIIIGILAAIA 27  
gi256760710 -----MRSHRGFTLIELMIVVAIIIGILAAIA 26  
gi94264560 -----MLQKKKQKGF<sup>T</sup>LVEVMIVVAIIIGILAMIA 30  
gi90411990 -----MKK-----VQKGF<sup>T</sup>LIELMIVVAIIIGILSAIA 27  
gi257455029 -----MN-----AQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 26  
gi207728014 -----MASKHSRNR<sup>R</sup>RGQRGF<sup>T</sup>LIELMIVVAIIIGILAAIA 35  
gil34298906 -----MLKIAKMLRN<sup>R</sup>KGF<sup>T</sup>LVELMVVAIIIGILATIA 33  
gi94309642 -----MKKARMGIRRVQKGF<sup>T</sup>LIELMIVVAIIIGILAAIA 34

\* \* \* \* \*  


Geobacter sulfurreducens IPQFSAYRVKAYNSAASSDLRNLKTALESFAFADDQTY----- 86  
Geobacter lovleyi IPQFTTYRIKGYNSNATSDLRNLKTVLESVFADRQGY----- 67  
Pelobacter carbinolicus DSM2380 IPQFAAYRQRAFNSAALTDINN<sup>L</sup>QKSEAAFFTDWQVFGHSA----- 72  
Geobacter uraniireducens Rf4 IPQFSKYRIQGFNASGNSDLKNIRTSQESLYAEWQH<sup>Y</sup>GLTQGLAT--VAG 78  
Geobacter FRC32 IPQFSAYRARGFNASQSDVRGLATSEAA<sup>L</sup>FGDAQVFGATQFIAAPVAPA 80  
Geobacter M18 IPQFSAYRAKAYNAA<sup>N</sup>SDLNKIKTGMEAYMADRQAY----- 67  
Geobacter metallireducens IPQFAAYRQKAFNSAAESDLKNTKTNLESYYSEHQFY----- 67  
Geobacter bemidjiensis IPQFSAYREKAYNAA<sup>N</sup>SDLNKFNKTGLEAFNADFQTY----- 67  
Geobacter M21 IPQFSAYRAKAYNSA<sup>N</sup>SDLNKMTGMEAYMADRQAY----- 67  
Pelobacter propionicus DSM2379 IPQFSAYRAKAYNSA<sup>N</sup>SDLNKIKTGMEAFMADNQQY----- 67  
Myxococcus xanthus DK1622 IPNFIK<sup>F</sup>QARSKQSEAKTNLKALYTAQKSFFSEKDRYSDFANEIG---- 77  
Neisseria gonorrhoeae MS11 IPSYQSYIEKGYQSQLYTEMVGINNVLKQFILKNPQDDNDILKSKLEIFV 79  
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Neisseria meningitides C311 LPAYQDYTARAQVSEAILLAEGQKSAVTEYYLNHGEWP<sup>G</sup>NNTS----- 70  
Shewanella baltica OS185 IPSFSEYLKQGRRFDAQQY<sup>L</sup>MTSVQALERNYSRQ<sup>G</sup>KYPAAQTLTN----- 75  
Shewanella ANA3 IPSFNEYLKQGRRFDAQQY<sup>L</sup>VSSAQALERHYSRNGLYPASQSLTN----- 72  
Shewanella MR4 IPSFNEYLKQGRRFDAQQY<sup>L</sup>VSSAQALERHYSRNGLYPASQSLTN----- 72  
Vibrio mimicus 603 VPQYQNYVKKSEAA<sup>S</sup>GLATLRLSLTTNIDTYIAD-----NGSF----- 68  
Pseudomonas aeruginosa PAO1 IPQYQNYVARSEGASALATINPLKTTVEESLSRGIAGSKIKIGTT---- 71  
Pseudomonas syringae IPQYNSYTLKAKYSEVVS<sup>M</sup>ANAYQTAVASCQETG--KKEGCDLT---- 69  
Pseudomonas aeruginosa PAK IPQYQNYVARSEGASALASVNPLKTTVEEALSRGWS-VKSGTGTE---- 64  
Pseudomonas aeruginosa Pal10594 LPAYQDYTVRTRVSEGLVLAEPAKLMISTDGSASTADLTRATT----- 69  
Pseudomonas aeruginosa pa5196 IPAYQDYITRGQVTEAVSLGGGLKSP<sup>L</sup>AEYGADKNAP<sup>T</sup>TLVAP----- 69  
Pseudomonas aeruginosa K122 IPAYQDYTARAQLSERMTLASGLKTKVSDIFSQDGSC<sup>P</sup>ANTAAT----- 71  
Acinetobacter calcoaceticus IPQYQKYTARSQITAGLAEISP<sup>G</sup>KTQFELALSEGTA----NSV----- 65  
Acinetobacter baumannii AYE IPQYQTYIAK<sup>S</sup>QVSRVSESGSLKTVIEDCLNNGKT----TVGE----- 66

Acinetobacter baumannii AB900	IPAYSNYQARGKLTAGLSEISAGKAAAEQKINDGSD-----	62
Acinetobacter baumannii ATCC1797	IPQYQKYTARSQITAAALAEISPGKTFELALSEGTA---NSV-----	65
Vibrio vulnificus	IPQYQNYVKKSEAAAGLATLRALTNNIDTFIAD-----TGSF-----	70
Desulfohalobium acetoxidans	IPQFASYRQKANSAAQSDLNKLTGMEAYNADNQDY-----	68
gi119899644	IPQFNEYRQKANDTSAIADAKNGITS-----	53
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gi94265584	IPQFAQYRERGFISSESDARNVATALEAHFASEQEYT-----	71
gi85860123	IPQFQQYRTRGYNTAAKADAKNAYTAAQAYFSDHPSV-----	70
gi218779223	IPNFIAIRKKAYDKAAMTDLHNLNQSILAYYTEE-GK-----	67
gi114776286	IPQFSNYRTKAFNAAAADANTGVTFEAFYTDNNQYPG-----	67
gi224369860	IPQFMYYKAKAYNAGSLSDLHNLRLFEFGYNATWDAYPN-----	73
gi258595733	IPQFAQYRIRGNSSALSDVRNLTTAQEAFFADWLRVAVTH-----	73
gi225199913	IPQFSAYQDRAKLGVITSDLRACLSESVAAADAVG--EDLEE-----	73
gi257092941	IPQFSDYTRKGEDKAAQSDARNILTS-----	53
gi256830926	IPQFTKYKRNAEASCEADLRNLCMNDAARFAVN--GTDNN-----	69
gi226225417	IPKFQDTKSKAYATSIKSDLNLTSMQESYFYHNETYANTAVSTG-----	74
gi85860121	LPQLQAYRIRGYNIQANSDAKNFYSSCLVDINQTSVD-----	67
gi258406253	IPQFTKYKASAMQSELEGNLSNCISILSAQFADN--GTAS-----	68
gi158520534	IPNFIAIRNKTFCSATSDANSIASAIADYFSIPAN-VSVTDGSL-----	79
gi257092940	IPQFVDYTRKGEDKAAQSDARNFLTA-----	52
gi14573312	IPQYQNYVSRQSVSRVMAEAGSLKTAVEACLQDGR-----AVGT-----	66
gi126165560	IPQYQDYTRTQVTRAVSEISALKTAESAILEGKK---LVSSD-----	67
gi31096010	IPQYQDYTRTQVTRAVSEISALKTAESAILEGKK---LVSSD-----	67
gi21464531	IPQYQDYTRTQVTRAVSEISALKTAESAILEGKK---LVSSD-----	67
gi121629638	IPQYQDYTRTQVTRAVSEISALKTAESAILEGKK---LVSSD-----	67
gi545130	IPQYQDYTRTQVTRAVSEISALKTAESAILEGKE---IVSTK-----	67
gi21629635	IPQYQDYTRTQVTRAVSEISALKTAESAILEGKE---IVSSK-----	67
gi120365	IPQYQDYTRTQVTRAVSEISALKTAESAILEGKE---IVSSA-----	67
gi218893619	IPQYQDYTRTQVTRAVSEISALKTAESAILEGKE---IVSSA-----	67
gi256832576	IPVFMNQRKQKAVDSSVRSDLRATQVEDFWVENQAY-----	72
gi262197337	IPAFMRYMNAKSSSEAEQMVKKVHDGAISYYHNPPTQSFQTPAKG-----	76
gi51245307	IPQYQNYTIKAKVGNALTSVDSLKTAVAVCAQEAAGILTTCTNTTT-----	75
gi188591293	IPQYQDYVTRSRWSSPLTEIAPLKQAIACQAVSGGNLTACGTLA-----	82
gi113866578	IPQYQDYVTRSRWSSPLTEIAPLKQAIACQAVSGGNLTACGTLA-----	82
gi14573309	IPQYQNYVSRQSVSRVMAEAGSLKTAVEACLQDGR-----AVGT-----	66
gi239813460	IPQYQTYVAKSQFTRAMGEAGSLKTAVDTCLLDGKT---VVG-----	72
gi149376813	IPQYQNYIARSQFSEHTLLGGARTAVQERVDQGFASFAVSTGTPN-----	77
gi14573315	IPQYQDYTRTQVTRAVSEISALKTAESAILEGKK---LVSSD-----	67
gi121534459	IPRFTTTADSANLAKMKADLRTIDSAMVYQAQNGS-----	70
gi151480	IPQYQNYVARSEGASALASVNPLKTTVEEALSRGWS-VKSGTGTE-----	70
gi120438	IPQYQNYVARSEGASALASVNPLKTTVEEALSRGWS-VKSGTGTE-----	70
gi88859017	IPQYQNYTINGANNACETQARALADKIS-----IGIHPAM-----	70
gi182625519	IPNFLAIQRKARVKADIATGKTIYDATAALIAEGKEGFNMPEKVI-----	76
gi168211645	IPNFLAIQRKARIQADIATGKTIYDATALIAEGKEGFNMPEKTT-----	76
gi110800846	IPNFLAIQRKARIQADIATGKTIYDATALIAEGKEGFNMPEKTT-----	79
gi545132	IPQYQNYVARSEGASALATINPLKTTVEESLSRGIAGENIKIGSQ-----	71
gi14573303	IPQYQNYVARSEGASALATINPLKTTVEESLSRGIAGSKIKIGTT-----	71
gi14573306	IPQYQNYVARSEGASALATINPLKTTVEESLSRGIAGSKIKIGTT-----	71
gi49075736	IPQYQNYVARSEGASALATINPLKTTVEESLSRGIAGSKIKIGTT-----	71
gi151463	IPQYQNYVARSEGASALATINPLKTTVEESLSRGIAGSKILIGTT-----	76
gi120405	IPQYQNYVARSEGASALATINPLKTTVEESLSRGIAGSKILIGTT-----	71
gi73540239	IPQYQDYVTRARWSDNITSIAPLKQAIACVQNN-AQAAIAAPCD-----	81
gi196158030	LPAYQTYTARATYSEVISASSAAKTAVEVCAQTGV--PADCTDIA-----	74
gi39704	IPQYQNYIARSQSVSRVMSSETGQMRTAIETCLLDGK-----	62
gi120390	IPQYQNYIARSQSVSRVMSSETGQMRTAIETCLLDGK-----	62
gi120383	IPQYQNYIARSQSVSRVMSSETGQMRTAIETCLLDGK-----	62
gi120388	IPQYQNYIARSQSVSRVMSSETGQMRTAIETCLLDGK-----	62
gi188996934	IPQYMYQRKAKVSSYAEPRIARACLMDLAAYCV---ENPSA-----	85
gi92114295	IPQYQNYVARSEVSAALATLKLQTPAELAIQE-----GRT-----	63
gi51245304	IPQYQNYTKRAKVTEGLNMAGAAKTAVSEYYQS---QNVLP-----	68
gi114331818	IPAYSQYQAKAKVTAGLTEIAAGKTAFAFSRVNNGDT-----	63
gi256760710	IPAYQAYVIRAQLTEGLSLMEGARIGIWDYYAQNGRFPANNGS-----	69
gi194264560	IPQFAQYRNMNVAASVEAAIKTCATELAASFAE---NGNTS-----	68
gi90411990	VPQYQKYVAKSEAASALATVAGLKTNIETAIIE-----TGTF-----	64
gi257455029	IPQYQNYIAKQSVSRVMSSETASVKTAIETCVNDGK-----	61
gi207728014	IPAYQDYTIRARVVEGLSLAAQAKALVVEN-AANAQSSALGS-----	77
gi134298906	VPYMNNTKDAQDAANEATARTLNGAISMMAKQTS-----	69
gi94309642	IPAYQDYVAKSKAASAYADIAAGKTAAYELMVVNPND-----G-----	71

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Geobacter sulfurreducens	-P-----PES-----	90
Geobacter lovleyi	-P-----GS-----	70
Pelobacter carbinolicus DSM2380	-----AAAAGAAPNAATAANAGAIVT-----GPDNGVAGN	102
Geobacter uraniireducens Rf4	LPGAGKGV---GALVTPAALP---VCIITT-----DDNNLVPRG	113
Geobacter FRC32	PPVFLAWAGGPGIMLLGPTGNPAGGAFIPSISA-----TDANGATRG	122
Geobacter M18	-P-----VSLDER-----	74
Geobacter metallireducens	-P-----N-----	69
Geobacter bemidjiensis	-P-----AAYVASTN-----	76
Geobacter M21	-P-----ALLDQR-----	74
Pelobacter propionicus DSM2379	-P-----GDVDYR-----	74
Myxococcus xanthus DK1622	FAPERGNRYGYRVSAAAGDCEVRNAADLPVPAAGVPCIS-----	116
Neisseria gonorrhoeae MS11	SG-----YKMNPKIAKKYSVSVRFVDAEKPRAYR-----	108
Neisseria meningitides Z2491	SG-----YKMNPKIAEKYNVSVHFVNEEKPRAYS-----	109
Neisseria meningitides C311	-----AGVATSSEIKGKYVKSVEVK-----	90
Shewanella baltica OS185	-----SPYYTFSYTPADDKLSFSL-----	95
Shewanella ANA3	-----NAYYSFSYTPTDKLGFSLK-----	92
Shewanella MR4	-----SAYYSFSYTPADKLGFSLK-----	92
Vibrio mimicus 603	-----PGTSDLPTLGAKDDMNKL-----	86
Pseudomonas aeruginosa PAO1	-----ASTATETYVGVEPDANKL-----	89
Pseudomonas syringae	-----KNS---IPDTQATTHVA-----	83
Pseudomonas aeruginosa PAK	-----DATKKEVPLGVAADANKL-----	82
Pseudomonas aeruginosa Pa110594	-----TWNQQSNNLGASSKYVTSV-----	88
Pseudomonas aeruginosa pa5196	-----TATPGAGQLNATLVGKYSSVDS-----	91
Pseudomonas aeruginosa K122	-----AGIEKDDTDINGKYVAKVTTGG-----	92
Acinetobacter calcoaceticus	-----NNSPAAIGLKSTTNCSA-----	83
Acinetobacter baumannii AYE	-----AAGECAIGATGSNILDGAAQ-----	86
Acinetobacter baumannii AB900	-----WTSPTDIGLQSTTSNCT-----	79
Acinetobacter baumannii ATCC1797	-----NNNPAAIGLKSTTNCSA-----	83
Vibrio vulnificus	-----PVDTDAAATLGASLNMNKL-----	88
Desulfuromonas acetoxidans	-----PAVLGFF-----	75
gi119899644	-----IISSLR-----	59
gi218780954	-----EINSFTLDNLK---DAGFKQT-----VGVA-----	90
gi94265867	-----HTCTGVDEGTTDPTVSIASD-----GTMSLENNT	103
gi226227929	-----SGSSGVTITPGTQS-----	85
gi94265584	-----DDLDDLVPVTTSGD-----NEVDITVAD	93
gi85860123	-----TISSIT-DLAN---YGFKAS-----ADVT-----	90
gi218779223	-----EGVVMTLDVAKTAKAGFRQT-----SNVT-----	91
gi114776286	-----ATTPATGSLAFGAGASLVTW-----TLSSGVTAG	96
gi224369860	-----EADVTEVKATGDLLEGPSTGAMVLAQWARQAHQQLPLAIGN	115
gi258595733	-----HCYIDGDDTTVENPFRDDTG-----GLENLEEIG	102
gi225199913	-----AVVNSSPE-----	61
gi257092941	-----QTCEIPGTLTGGDFSVQNN-----GTIRFVTGN	98
gi256830926	-----FISSKGVNIVIAESN-----	89
gi226225417	-----KTFSYP-NPLP---SGYHGT-----TPFSGS---	89
gi85860121	-----YDCSLGDSTVT---LEIGSD-----GLVDEAT-N	93
gi258406253	-----ESDANG---KTYVGTELSNGNTWDVDATNMDNIVIEVD	115
gi158520534	-----ALLVNSGE-----	60
gi257092940	-----AAGQCDPGATGSSLLTGASQ-----	86
gi14573312	-----TPGNNEYDLGFTSSTLLTGS-----	87
gi126165560	-----SPKNDEYDLGFTSTRSLLTGD-----	87
gi31096010	-----SPKNDEYDLGFTSSTLLTGS-----	87
gi21464531	-----NPADGEYDLGFTKSTLLAGND-----	88
gi 21629638	-----TPNDTQYDIGFTESTLLDLK-----	87
gi545130	-----TPKTDQYDIGFTGSTLLKGS-----	87
gi21629635	-----TPKTDQYDIGFTESTLLDGS-----	87
gi120365	-----TPKTDQYDIGFTESTLLDGS-----	87
gi218893619	-----PTDQTEFDAMGVATST-----GNTFVYTN-	96
gi256832576	-----FPITQALTPTAACCPGVQEKCPADA-----	102
gi262197337	-----ADTVTEIPAFTPTKEAL-----	92
gi51245307	-----QLTAATGYPAVPTPAGVTLLFGAGA-----	107
gi188591293	-----QLTAATGYPAVPAPAGVTLLFGAGA-----	107
gi113866578	-----AAGQCDPGATGSSLLTGASQ-----	86
gi14573309	-----AAECNPAASASTILVGAAQ-----	92
gi239813460	-----ATTNVLGLQLNGEYGTMAAPAYN-----	100
gi149376813	-----SPKNDEYDLGFTSSTLLTGS-----	87
gi14573315	-----YPATINNLVS-AKLLAGVPAPPKKYKGFVKQYED	104
gi 121534459	-----DATKKEVPLGVAADANKL-----	88
gi151480	-----DATKKEVPLGVAADANKL-----	88
gi120438	-----AMTAAEY-----	77
gi88859017	-----TEDSEGHVEY-GYGIDLSRN-DGMAKILKEYISNNTN	112
gi182625519		

gi 168211645	-----IKDKDGKLITYYGYGIDLSSSSDERAKELRKYILNSTN	114
gi110800846	-----IKDKDGKLITYYGYGIDLSSSSDERAKELRKYILNSTN	117
gi545132	-----ASTADTTYVGVDATANKL-----	89
gi14573303	-----ASTATETYVGVEPDANKL-----	89
gi14573306	-----ASTATETYVGVEPDANKL-----	89
gi49075736	-----ASTATETYVGVEPDANKL-----	89
gi151463	-----ASTADTTYVGIDEKANKL-----	94
gi120405	-----ASTADTTYVGIDEKANKL-----	89
gi73540239	-----DATAATG--LVSTAGGG--YTAGV-----	101
gi196158030	-----VNAGWTNASTVNTIEIG-----	91
gi39704	-----EGKDCFIGWTTSNLLAAAGG-STT-----	85
gi120390	-----KADECFIGWTKSNLLGASGSPSSS-----	86
gi120383	-----EAGECFIGWTKSNLL--DGDFTAG-----	84
gi120388	-----EGKDCFIGWTTSNLLAAAGG-STT-----	85
gi188996934	-----TSLIDSTIAKNCTTTTTPG-----GTVNLNGGG	114
gi92114295	-----ISDDLSELGLSSDSVQN-----	81
gi51245304	-----TSLAEAGISTVDTDIVKS-----	86
gi114331818	-----VSAIGDVGLTTPTSNCN-----	80
gi256760710	-----IHLPSPGSISGHYVSGLN-----	87
gi 94264560	-----HACSGVAAGVT--LALTDG-----GTFVAVNDT	94
gi90411990	-----PSLTATDAGNAQYGVPKVTT-----	84
gi257455029	-----QAADCDIGWTKSNLL-----	76
gi207728014	-----ASLPASKNVSALNIDASTGEIAVV-----	101
gi134298906	-----TEVAAFVVKDKAGVLAELVAKGYIQAGADVTN-LN	103
gi94309642	-----TPTFTPAGIGLQIATGNCS-----	90

Geobacter sulfurreducens	-----	
Geobacter lovleyi	-----	
Pelobacter carbinolicus DSM2380	VIWVTGNAQSLQAGLSNNVDMIAHTNAGGDSFNAATKHVQGSRSYAVDAD	152
Geobacter uraniireducens Rf4	LQIPVGN---NVTAMATT--AAAGAGDGGSYTLAAKHLQGDVIFAADSD	157
Geobacter FRC32	IQIPLGN---NVDIVAGTNLAAAAILANTTFTAVSKHVTGNTYFGVDGD	168
Geobacter M18	-----	
Geobacter metallireducens	-----	
Geobacter bemidjensis	-----	
Geobacter M21	-----	
Pelobacter propionicus DSM2379	-----	
Myxococcus xanthus DK1622	-----NDSFRFGANSALDDPTPVVARFVPQGA	144
Neisseria gonorrhoeae MS11	-----LVGVPNAGTGTYTLVWMNSVGDYKCRDAT	138
Neisseria meningitides Z2491	-----LVGVPKTGTYTLVWMNSVGDYKCRDAA	139
Neisseria meningitides C311	-----NG--VVTATMLSSGVNKEIKGKK	111
Shewanella baltica OS185	-----AVPTSRQSDSCGTLINQKGVRTPA	121
Shewanella ANA3	-----AVPTSRQSDSCGTLSLDHKGVRAPAT	118
Shewanella MR4	-----AVPTSRQSDSCGTLSLDHKGVRAPAT	118
Vibrio mimicus 603	-----GTISLA--DSGASGATATFTFDGTA	109
Pseudomonas aeruginosa PAO1	-----GVIAVAIE--DSGAGDITFTFQTGTS	113
Pseudomonas syringae	-----SVAVKDGTITVTPTATT---IAAAT	105
Pseudomonas aeruginosa PAK	-----GTIALKPDADGTADITLFTTMGA	107
Pseudomonas aeruginosa Pal10594	-----LMDAGNTGVITITYVADQVGLPTAGNT	115
Pseudomonas aeruginosa pa5196	-----TIASGYPNGQITVTMTQGA---SGKK	115
Pseudomonas aeruginosa K122	-----TAAASGGCTIVATMKASDVATPLRGKT	119
Acinetobacter calcoaceticus	-----VNVTANGT--TGTIACTLQGS-A	103
Acinetobacter baumannii AYE	-----SGETLAAG--TGVPQVTLANTG---AATIVATFGNSAS	119
Acinetobacter baumannii AB900	-----ITSTMSAG--AGTIKCEVLNAPS	100
Acinetobacter baumannii ATCC1797	-----VNVTANGT--TGTIACTLQGS-A	103
Vibrio vulnificus	-----GTLSTFA--DSGASGATATFTFNAGTA	111
Desulfuromonas acetoxidans	-----	
gi119899644	-----	
gi218780954	-----ITVTS-VNFQDFELTARHGQGDIVYTIDAQ	119
gi94265867	G-----MTVSGYTIDCTLDNG	119
gi226227929	-----ATGWSATATHANA-----AGSSCEIGVGTG	110
gi94265584	-----DGNSFT--VEVTSARTEKEVFTSDDG	118
gi85860123	-----TTAAGDMGLAITAKHSASDTTYQVDSQ	118
gi218779223	-----VTVDGGTGQNDWTITTKHGQGDKTYTMTAN	121
gi114776286	SSTNNGGT-----AGQNYMLMTKKHVAGDNCYSATDT	126
gi224369860	-----	
gi258595733	GVVMQADVIPATAVSYYVAISKHLQGNMTMYGATNTSTAIHRDQETLVPQGQ	165
gi225199913	-----GDDGTLTVDGTAHDDIENDVECDFDGR	129
gi257092941	-----	
gi256830926	-----QYYSAQNTLN-YAGYDFYVQID-EGA	123
gi226225417	-----GSGWSASATHPSS-----IPLTCAVYYGAA	114

gi85860121	-----FIYVAATGTVTCNAAFKHPNGTKTYALDNN	119
gi258406253	-----STDLSNIAVESYDDANCTLTNDNDVT	120
gi158520534	VSGR-----CPDDYQTAMDATVS--PRGYWDGN	141
gi257092940	-----	
gi14573312	-----TSQTLPTN--TGVPQVLDPLTT---QTTIIATFGNGAS	119
gi126165560	-----GKGQIKIDKADT--ATPEISGTLGNSSG	113
gi31096010	-----GKGQIKIDKADT--ATPEISGTLGGSSG	113
gi21464531	-----GKGQIKIDKADT--ATPEISGTLGGSSG	113
gi 21629638	-----GKAQITITGESS--ATPTIAGTLGNSAG	114
gi545130	-----GKEQIKVTDNKN--GTVQLVATLGGSSG	113
gi21629635	-----EKGHITVKNGTL--PTVQLEATLDGSSG	113
gi120365	-----GKSQIQVTDNQD--GTVELVATLGKSSG	113
gi218893619	-----GKSQIQVTDNKD--GTVELVATLGKSSG	113
gi256832576	-----DQSYTIVGTNDNGDAASTAEGG	119
gi262197337	-----AEEAEWAASNTWKSLSNFSMDSPHYQYEFDLN	134
gi51245307	-----TGTVAAGIITLTLEDGIGTGVDDL	117
gi188591293	-----PTAPTATTTTIELTATANNLGAANGCV	134
gi113866578	-----PTAPTATTTTIELTAAANNLGLANGCV	134
gi14573309	-----TSQTLPTN--TGVPQVLDPLTT---QTTIIATFGNGAS	119
gi239813460	-----AGAPAVTTGVNYPQVSLGATAA--AASTVIATLGNSSA	129
gi149376813	-----GSDATYALTYTFAASDVNANLQNET	125
gi14573315	-----GKGQIKIDKADT--ATPEISGTLGGSSG	113
gi 121534459	LNED-----ASSPNYKRAYVTITGIGYYSDST	132
gi151480	-----GTIALKPPDPADGTADITLFTTMGGA	113
gi120438	-----GTIALKPPDPADGTADITLFTTMGGA	113
gi88859017	-----TAMTASTTACESILPA---ALPTAFP	100
gi182625519	LKPQ-----SAKDCHFVYNVEFGKSSDGSNDGN	140
gi 168211645	LKPK-----AFKGCHFVSVTFDFSDNNSNNGY	142
gi110800846	LKPK-----AFKGCHFVSVTFDFSDNNSNNGY	145
gi545132	-----GTIAVTIG--NNGAGTVTFTFAAGNS	113
gi14573303	-----GVIAVAIE--DSGAGDITFTFQSGTS	113
gi14573306	-----GVIAVAIE--NSGAGDITFTFQSGTS	113
gi49075736	-----GVIAVAIE--DSGAGDITFTFQSGTS	113
gi151463	-----GTVAVTIK--DTGDGTVKFTFATGQS	118
gi120405	-----GTVAVTIK--DTGDGTVKFTFATGQS	113
gi73540239	-----PTPSSATVVTYTGGVITLTGTAAAGNS	128
gi196158030	-----GDAANGYTITVTPNEESGI--VAADT	115
gi39704	-----NNATAEDPGQGLNITYALGST---AENKIEATFGQNAA	121
gi120390	-----NDSTADHPGQGLVIDYKLEAD---ATNAITATFGQNAA	122
gi120383	-----TESTAAATGQAGITIKYPVAAD---DEGNIVATFGRNAA	120
gi120388	-----NNATAADPGQGLNITYALEST---AENKIEATFGQNAA	121
gi188996934	FV-----CDNTGSITAGS--VTATLGNISDYTASCT	143
gi92114295	-----GTIGLSDFDGEDDSTPSIVFTFDE	106
gi51245304	-----LGIGASGVITVTFKAPVL---LDAT	108
gi114331818	-----ISVTD-----TIVCELIHAPS	97
gi256760710	-----VSNPGSGVLVTVTYGGKANSIQGKT	113
gi 94264560	-----INVGNNTVAWSIDAA	109
gi90411990	-----GTIAFVGAEDAANGAVNYLFTKGKA	109
gi257455029	-----VGATAAATGQTGLAITYPLKADGTFQEGKIEGTFGGNAS	115
gi207728014	-----YATAVTAGGANTLVLTTPYSGGS--SAFAN--	128
gi134298906	FTDG-----TATPLKAPVYVAP-----	120
gi94309642	-----AIAVAAPDNTGAATNAIQCTIAAP	114

Geobacter sulfurreducens	-----	
Geobacter lovleyi	-----	
Pelobacter carbinolicus DSM2380	LTATYMAEATVGNNLVAGDIINPVAQNDDFNGQNNNDGNPFTMM-----	196
Geobacter uraniireducens Rf4	STANYKMTFA----APATGLNAGYPLTAAFPVSVNNVDDYQALPNWVKM	203
Geobacter FRC32	TTAIYMKAAAGSEGTPIPALGAGVLPASTTGDDFSGVAGPGLPAGNWAAC	218
Geobacter M18	-----	
Geobacter metallireducens	-----	
Geobacter bemidjiensis	-----	
Geobacter M21	-----	
Pelobacter propionicus DSM2379	-----	
Myxococcus xanthus DK1622	GWNTTLGVQPTIADCPNCNFFAGARGNADNEATFDDWWIAGFEGSGQVGP	194
Neisseria gonorrhoeae MS11	SAQVYLETL SANTGCEAFSNRKK-----	161
Neisseria meningitides Z2491	SARAHLETLSDDVGCEAFSNRKK-----	162
Neisseria meningitides C311	LSLWA-----KRQN-----GSVKWFCGQPVTRNDTDD	138
Shewanella baltica OS185	NCWTN-----	126
Shewanella ANA3	HCWTH-----	123
Shewanella MR4	HCWTH-----	123

Vibrio mimicus 603	SALANS-----DTVVLT KDSTT-----GLWTC-----	131
Pseudomonas aeruginosa PAO1	SPKNAT-----KVITLNRT-AD-----GVWACK-----	135
Pseudomonas syringae	YVVTP-----VIGAGAVTWTQSGGCL-----	126
Pseudomonas aeruginosa PAK	GPKNKG-----KIITLRTAAD-----GLWKCT-----	130
Pseudomonas aeruginosa Pa110594	LILSPYINDGNTRTALATAVAAGT-----RGTDWACTS-----ASNATA	155
Pseudomonas aeruginosa pa5196	LTFST-----QDGG-----SSWACGN-----	131
Pseudomonas aeruginosa K122	LTLTL-----GNADK-----GSYTWAC-----	136
Acinetobacter calcoaceticus	TITGG-----ILTLTRSADVAAASGVNANVGGWTC SITK-----	137
Acinetobacter baumannii AYE	TALKSTP-----TTVTWTRTTD-----GTWTCEST-----	144
Acinetobacter baumannii AB900	QVRGA-----AITWTR-----DKAGTWSCATS-----	122
Acinetobacter baumannii ATCC1797	TITGG-----VLTLTRSADVAAASGVNANVGGWTC SITK-----	137
Vibrio vulnificus	SALQST-----DTVVL SKDATT-----GLWTC-----	133
Desulfuromonas acetoxidans	-----	
gi119899644	-----	
gi218780954	GLRTQN-----	125
gi94265867	VLSCDEQGA-----	128
gi226227929	KPANLAEGEP-----GGATCK-----	126
gi94265584	QLVTE-----	123
gi85860123	GTITP-----	123
gi218779223	QTLTVD-----	127
gi114776286	APSIATTTGGTKGSVLATVGTC P-----	149
gi224369860	-----	
gi258595733	GDVLPITGYMPEPHETDDPFIDHEEF EAQ-----	194
gi225199913	SLRDCVYAD-----	138
gi257092941	-----	
gi256830926	NANCTLAP-----	131
gi226225417	APLAPATRE-----GVIECQ-----	129
gi85860121	GNISITGS-----	127
gi258406253	DIDCTVE-----	127
gi158520534	SLYVKSMTME-----	151
gi257092940	-----	
gi14573312	AAISG-----QTLTWTRDVN-----GGWSCATT-----	142
gi126165560	KGIAG-----AVITVKRDDK-----GVWTCGITG-----	137
gi31096010	KGIAG-----AVITVKRDDK-----GVWTCGITG-----	137
gi21464531	KGIAG-----AVITVKRDDK-----GVWTCGITG-----	137
gi121629638	KAISG-----AVITIKRSAE-----GVWTCATSG-----	138
gi545130	SAIKG-----AVITVSRDAQ-----GVWSCNITK-----	137
gi21629635	AAIKG-----AVITVSRAD-----GVWGCSISS-----	137
gi120365	SAIKG-----AVITVSRKND-----GVWNCKITK-----	137
gi218893619	SAIKG-----AVITVSRKND-----GVWNCKITK-----	137
gi256832576	ITYN-----SAAGGLQP-----	131
gi262197337	DPAGELTGSPLFVAGAEGDLDCDGV TGRFEMGA EVTADGLKSSSAIRRT	184
gi51245307	ITLTP-----TIGTTTTITWNNTTTVT-----	138
gi188591293	VTLTG-----TSNANSIVWTGATTG-----	154
gi113866578	VTLTG-----TANANSIVWTGVTG-----	154
gi14573309	AAISG-----QTLTWTRDVN-----GGWSCATT-----	142
gi239813460	QPLKT-----KITWSRAGDS-----GTWTCSTN-----	153
gi149376813	VTTYTY-----TAASG-----EWD CVT-----	141
gi14573315	KGIAG-----AVITVKRDDK-----GVWTCGITG-----	137
gi121534459	DWP-----	135
gi151480	GPKNKG-----KIITLRTAAD-----GLWKCT-----	136
gi120438	GPKNKG-----KIITLRTAAD-----GLWKCT-----	136
gi88859017	VTLTA-----TPKAP-----GTG-----	113
gi182625519	DLNKPIIQVSIINSNND SIGDVFPNTDEDFK-----	171
gi168211645	DLTKPIVKVDIVNSDNKSKGEAYPN-NELLK-----	172
gi110800846	DLTKPIVKVDIVNSDNKSKGEAYPN-NELLK-----	175
gi545132	SPKNSG-----AIITLRT-DA-----GSWACT-----	135
gi14573303	SPKNAT-----KVITLNRT-AD-----GVWACK-----	135
gi14573306	SPKNAT-----KVITLNRT-AD-----GVWACE-----	135
gi49075736	SPKNAT-----KVITLNRT-AD-----GVWACK-----	135
gi151463	SPKNAG-----KEITLNRT-AE-----GVWTCT-----	140
gi120405	SPKNAG-----KEITLNRT-AE-----GVWTCT-----	135
gi73540239	VTVTP-----VPSATAVTWTVANQAG-----	149
gi196158030	LILTG-----TINNGAVTWATTGGCV-----	136
gi39704	ATLHG-----KKLTWTR SPE-----ATWSCSTD-----	144
gi120390	ATLHG-----KALKWTRDPK-----ATWSCSTD-----	145
gi120383	AAIKP-----QTLTWSRSKE-----GTWTCATT-----	143
gi120388	ATLHG-----KKLTWTR SPE-----ATWSCSTD-----	144
gi188996934	VINNGFKCTVQ-----	154
gi92114295	G-NFSN-----ETLTLERQ-DD-----GGWECSAS-----	129
gi51245304	IVLTP-----SLSTTGAG-----LTWTC TN---TG-----	130

gi114331818	QVISK-----KITLTRA-----ADTGVWSCAT-----	119
gi256760710	FYLSG-----VAGAN-----SITWRCTN---APGGTS	137
gi194264560	GAVVASQP-----	117
gi90411990	SVELVA-----KTLTLTRD-TS-----GNWACTVNF-----	134
gi257455029	GAIGT-----KKLTWTRDTA-----GSWACTTN-----	138
gi207728014	-LHAG-----SAPPA-----MVLWVCA---AAGKAM	150
gi134298906	-----	
gi94309642	GRLGTAP-----VSIQFNRLAT-----GLYTCTTTG-----	140

Geobacter sulfurreducens	-----	
Geobacter lovleyi	-----	
Pelobacter carbinolicus DSM2380	-----	
Geobacter uraniireducens Rf4	-----	
Geobacter FRC32	-----	
Geobacter M18	-----	
Geobacter metallireducens	-----	
Geobacter bemidjensis	-----	
Geobacter M21	-----	
Pelobacter propionicus DSM2379	-----	
Myxococcus xanthus DK1622	CSEAGNVASGTPYNTRNDVACDGAAG--- 220	
Neisseria gonorrhoeae MS11	-----	
Neisseria meningitides Z2491	-----	
Neisseria meningitides C311	TVAAVAADNTGNINTKHLPSSTCRDASDAS- 167	
Shewanella baltica OS185	-----	
Shewanella ANA3	-----	
Shewanella MR4	-----	
Vibrio mimicus 603	-----STTTGVTLKGC----- 142	
Pseudomonas aeruginosa PAO1	-----STQDPMFTPKGCDN----- 149	
Pseudomonas syringae	-----TSTPILCK----- 134	
Pseudomonas aeruginosa PAK	-----SDQDEQFIPKGCSR----- 144	
Pseudomonas aeruginosa Pa110594	TAQGFTGMAAGSVQEFAPAQCR----- 178	
Pseudomonas aeruginosa pa5196	ASIDGFA GTGTIDAKYLPNACKP----- 155	
Pseudomonas aeruginosa K122	-----TSNADNKYLPKTCQTATTTTP 157	
Acinetobacter calcoaceticus	-----GTDDISSAIAPKGCTII----- 154	
Acinetobacter baumannii AYE	-----AAEKYNSSACPAA----- 157	
Acinetobacter baumannii AB900	-----GLDDDKDLAPKSCPQS----- 139	
Acinetobacter baumannii ATCC1797	-----GTDDISSVIAPKGCTII----- 154	
Vibrio vulnificus	-----SHTTGVTLKGC----- 144	
Desulfuromonas acetoxidans	-----	
gi119899644	-----	
gi218780954	-----	
gi94265867	-----	
gi226227929	-----	
gi94265584	-----	
gi85860123	-----	
gi218779223	-----	
gi114776286	-----	
gi224369860	-----	
gi258595733	-----	
gi225199913	-----	
gi257092941	-----	
gi256830926	-----	
gi226225417	-----	
gi85860121	-----	
gi258406253	-----	
gi158520534	-----	
gi257092940	-----	
gi14573312	-----VDAKFRPNGCTD----- 154	
gi126165560	-----SPTNWKTNYPANCPKS----- 154	
gi31096010	-----SPTNWKANYAPANCPKS----- 154	
gi21464531	-----SPTNWKANYAPANCPKS----- 154	
gi121629638	-----SPANWKANYAPANCPKS----- 155	
gi545130	-----TPTAWKPNYPANCPKS----- 154	
gi21629635	-----TPANWKPNYAPSNCPKS----- 154	
gi120365	-----TPTAWKPNYPANCPKS----- 154	
gi218893619	-----TPTAWKPNYPANCPKS----- 154	
gi256832576	-----	
gi262197337	NENE----- 188	
gi51245307	-----QSAALAAI IKNNI----- 151	
gi188591293	-----ATCTRAQTGI----- 164	

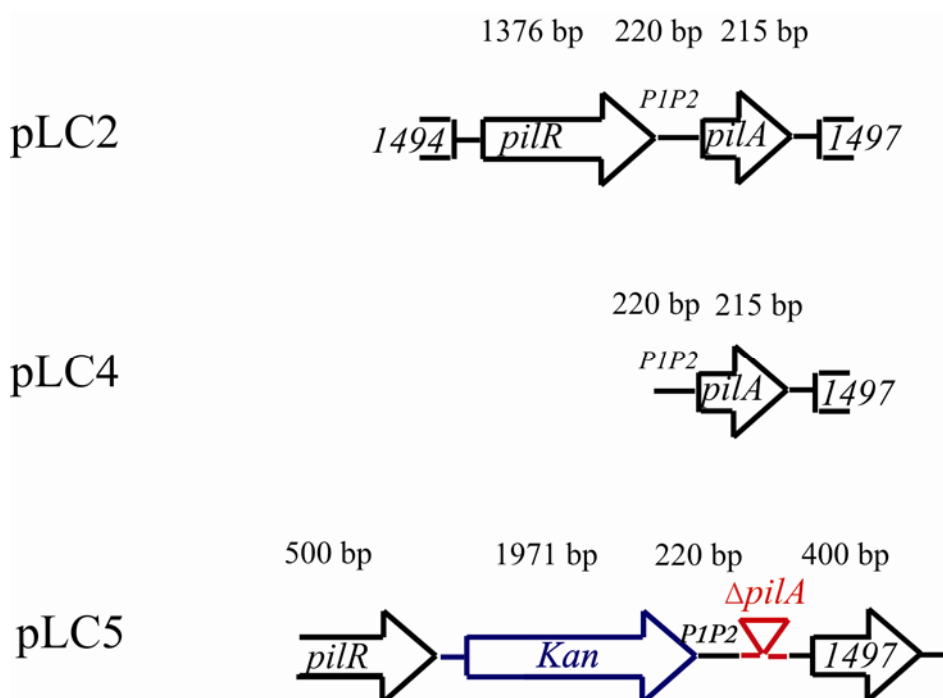
gi113866578	-----ATCTRAQTGI-----	164
gi14573309	-----VDAKFRPNGCTD-----	154
gi239813460	-----ADQKYVPTGCTVVP-----	167
gi149376813	-----TVEEQFASQCTSS-----	154
gi14573315	-----SPTNWKANYAPANCPKS-----	154
gi 121534459	-----	
gi151480	-----SDQDEQFIPKGCSK-----	150
gi120438	-----SDQDEQFIPKGCSR-----	150
gi88859017	-----TITITR-----	119
gi182625519	-----	
gi 168211645	-----	
gi110800846	-----	
gi545132	-----STQDEMFIPKGCKAP-----	150
gi14573303	-----STQDPMFIPKGCDN-----	149
gi14573306	-----STQDPMFIPKGCDN-----	149
gi49075736	-----STQDPMFIPKGCDNG-----	150
gi151463	-----STQEEMFIPKGCNKP-----	155
gi120405	-----STQEEMFIPKGCNKP-----	150
gi73540239	-----GAVVCTKSKTGV-----	161
gi196158030	-----DKG--LC-----	141
gi39704	-----VDEKFKPTGCKQ-----	156
gi120390	-----VELKFRPTGCKDDLK---	160
gi120383	-----VEAKFQPTGCKDK-----	156
gi120388	-----VDEKFKPTGCKK-----	156
gi188996934	-----	
gi92114295	-----QGIDDDVVPDSCVANSN---	146
gi51245304	-----TDMDN-----YVPANCR-----	142
gi114331818	-----DANNKYAPKSCPGNGS---	135
gi256760710	PATAV-----PAQYLPASCR-----	152
gi 94264560	-----	
gi90411990	-----G-SSTD TALIPKNCIFQ-----	150
gi257455029	-----VDAKYKPTSCG-----	149
gi207728014	PVTTVTQNTTATLPPKYAPAECR-----	173
gi134298906	-----	
gi94309642	-----ITNTSF IPTGCVAG-----	154



## APPENDIX E

### LIST OF PLASMIDS GENERATED DURING THE PH.D. RESEARCH COURSE BUT NOT USED IN ANY OF THE CHAPTERS

Plasmids	Description
pLC2	Plasmid containing the entire GSU1495 ( <i>pilR</i> ) gene, the P1 and P2 promoter regions and the entire GSU1496 ( <i>pilA</i> ) gene cloned in the pLC1 vector (see Chapter 2 for details on pLC1). Spec <sup>r</sup>
pLC4	Plasmid containing the P1 and P2 promoter regions and the entire GSU1496 ( <i>pilA</i> ) gene cloned in the pLC1 vector (see Chapter 2 for details on pLC1). Spec <sup>r</sup>
pLC5	Plasmid containing the last 500 bp of GSU1495 ( <i>pilR</i> ), the kanamycin resistance cassette, the P1 and P2 promoter regions, the first ten and last ten codons of <i>pilA</i> fused by annealing of an overlapping sequence of 24 base pairs (codons 11 to 18 of <i>pilA</i> ), and the first 400 bp of GSU1497 cloned in the pLC1 vector (see Chapter 2 for details on pLC1). Spec <sup>r</sup> and Kan <sup>r</sup>



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